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Investigating RNA silencing and mycoviruses in *Botrytis cinerea*

Could host-mediated gene silencing prevent the use of mycoviruses for the biological control of *Botrytis cinerea*?

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Joseph

School of Biological Sciences

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A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the Faculty of Science

Abstract

The haploid fungus *Botrytis cinerea*, teleomorph: *Botryotinia fuckeliana* (Whetzel, 1945) is the cause of the gray mould disease. It is an airborne plant pathogen with a necrotic lifestyle attacking a large number of economically important vegetable, flower and fruit crops.

For the control of *B. cinerea*, cultural control measures and fungicides are both used. The use of biological control for *B. cinerea* has become of interest since *Cryphonectria parasitica* was able to be controlled by the hypovirulent mycovirus CHV1. If the use of mycoviruses as a possible control for *B. cinerea* is to be considered, more has to be understood of the RNA silencing mechanism which acts as a cellular defence system against virus infection. RNA silencing has been reported to protect the host against exogenous (e.g. viruses) and endogenous RNA elements. The DICER protein is an evolutionary conserved RNase III enzyme which initiates RNA silencing by recognizing and cleaving exogenous and endogenous double stranded RNA into 21 bp short interfering RNA.

Bioinformatic analysis identified two likely Dicer genes in *B. cinerea* (*Dcr1* and *Dcr2*). For most fungi, it has been reported that of the two Dicer genes present, only Dicer 2 is involved in RNA silencing. Therefore, *B. cinerea* *Dcr2* was targeted for gene disruption. Two *B. cinerea* *Dcr2* mutants (BcDcr2-1, BcDcr2-2) were made in a *ku70* background. RNA silencing assays showed that both *Dcr2* mutants were still capable of silencing. Real time PCR (qPCR) results showed no compensatory response of *Dcr1* expression due to the loss of *Dcr2*.

The sequenced single stranded RNA mycovirus *Botrytis virus F* (BVF) was transfected into *B. cinerea* lines to determine whether the mycovirus had any effect on host morphology and physiology. *Botrytis cinerea* lines infected with BVF showed no change in virulence in pathogenicity assays, while qPCR results showed a suppression of *Dcr1* and *Dcr2* expression at 7 days and recovery by 28 days post-infection. Argonaut 1 (*Argo1*) expression was increased during viral infection at both time points.

This study has identified two Dicer genes in *B. cinerea*, and suggests that *Dcr2* is not essential for RNA silencing. Also, the presence of BVF altered the expression of the Dicer genes in *B. cinerea*, showing that there was a possible interaction between the mycoviruses and the RNA silencing machinery.

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Author's declaration

I declare the work in this dissertation was carried out in accordance with the regulations of the University of Bristol. The work is original, except where indicated by special reference in the text, and no part of the dissertation has been submitted for any other academic award, any views expressed in the dissertation are those of the author.

Signed 

Seuseu Tauati

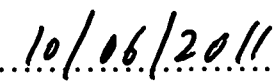
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Abbreviations

<i>Argo1</i>	Argonaut one gene
BCA	Biological control agent
Basta	Glufosinate ammonium herbicide
BLAST	Basic local alignment search tool
Bcass1	<i>Botrytis cinerea</i> argininosuccinate synthase gene
bp	Base pair
BVF	<i>Botrytis</i> virus F
BVX	<i>Botrytis</i> virus X
cDNA	Complimentary DNA
<i>Dcr1</i>	<i>B. cinerea</i> Dicer one gene
<i>Dcr2</i>	<i>B. cinerea</i> Dicer two gene
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
MEA	Malt extracted agar media
miRNA	Micro RNA
mRNA	Messenger RNA
NCBI	National centre for biotechnology information
PCR	Polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
siRNA	Short interfering RNA

ssRNA	Single stranded RNA
TAE	Tris base and acetic acid and EDTA
qPCR	Quantitative PCR

1 Introduction

1.1 *Botrytis cinerea*

The haploid fungus *Botrytis cinerea*, teleomorph: *Botryotinia fuckeliana* (Whetzel, 1945) is an airborne plant pathogen with a necrotic lifestyle reviewed by Williamson *et al.*, (2007). It can attack over two hundred crop species worldwide including a large number of economically important vegetable, flower and fruit crops where it causes gray mould disease (Fig 1.1), so named because of its prolific sporulation, producing high numbers of gray conidia. The fungus is responsible for both pre- and post- harvest rots on a variety of fruits such as strawberry, raspberry, apple, pear, kiwifruit and grapefruit (Castro, 1999). *B. cinerea* has caused harvest losses up to 50 % for cocoa (Perry, 1990) and onion (Schwartz and Bartolo, 2004), and 8 million pounds sterling in losses to the kiwifruit industry in New Zealand in just one year (Garnham, 1996). Chemical control for *B. cinerea* costs in excess of 500 million pounds sterling in 2001 (Annual Report, University- Industry Partnership Project, 2002).

Botrytis cinerea has a worldwide distribution but prefers relatively high humidity and an optimal temperature ranging from 20 - 25°C (Howitt *et al.*, 2001). These conditions are typically found in temperate geographical regions and also in poorly ventilated greenhouses. Despite these clear preferences, it has been reported that the fungus can be found in temperatures between 0°C and 33°C and can withstand substantial airflow (Brooks and Cooley, 1917) such as the cold temperatures of Alaska (Anderson, 1924) and dry climates of Israel (Yunis and Elad, 1989).

There are over 20 *Botrytis* strains named in the literature (Staats *et al.*, 2005), however, with no simple key to distinguish between the strains, genetic analysis has been utilised. Phylogenetic analysis has used sequences that encode DNA dependent RNA polymerase subunit II (RPB2), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and the heat shock protein 60 (HSP60) in addition to the more commonly used ribosomal RNA regions to classify the different strains (Staats *et al.*, 2005). The resulting phylogenetic tree divided *Botrytis* into two clades; clade 1 with 4 species including *B. cinerea* from dicotyledonous hosts and clade 2 with 18 species, 3 from dicotyledonous hosts and 15 from monocotyledonous hosts (Staats *et al.*, 2005). *Botrytis cinerea* has a wide host range compared to the other pathogenic *Botrytis* species which usually have a host range restricted to one or two monocotyledon or dicotyledon host species (Beever and Weeds, 2004). The most recent *Botrytis* species to be identified was *B. fabiopsis* in China which caused chocolate spot of broad bean (Zhang *et al.*, 2010).

Botrytis cinerea has a simple life cycle (Fig 1.2). The fungus is able to overwinter as mycelia or as sclerotia (mass of hardened mycelia) (Coley *et al.*, 1980). Sclerotia develop within dying host tissue and may not be readily apparent in susceptible crops (Williamson *et al.*, 2007). With favourable conditions, normally early spring in temperate regions, mycelia or sclerotia develop conidiophores which are branching structures bearing numerous single celled conidia. The conidia are dispersed into the air currents by the twisting and drying of the conidiophores caused by the early morning rapid decline in humidity and rise in temperature (Jarvis, 1962). Mycelia that germinate from conidia normally cannot penetrate the cuticle, however, on susceptible host plants, mycelia can enter through natural openings, wounds or particularly susceptible tissues such as petals (Cotoras and Silva, 2005).

Botrytis cinerea has also been reported to use enzymes such as cutinases and lipases to help it gain entry into the host (Salinas, 1992; Commenil *et al.*, 1998). Inside the host plant, mycelia proliferate and invade the plant tissue causing collapse and disintegration of the cells (Mendgen and Deising, 1993). Conidiophores then develop from the mycelia producing more conidia, and the cycle continues. The summer cycle would take 2- 4 weeks with the winter cycle taking longer.

Symptoms typical of *B. cinerea* infection include blight and soft rot (Hausbeck and Moorman, 1996). Infected leaves and flowers develop chocolate-coloured lesions, buds may fail to open while stems may wilt and fall over (Beever and Weeds, 2004). Soft rots on fruits and leaves are usually caused by the collapse of the parenchyma cells followed by the formation of the typical *B. cinerea* gray conidia (Williamson *et al.*, 2007). Infection of thicker skinned fruits may go unnoticed until cut, showing macerated and dark coloured soft tissue below the skin.

Botrytis cinerea can also grow as an endophyte, being present in the host without showing any apparent symptoms. This has been seen for lettuce where *B. cinerea* has entered through the flowers and grown systemically through the plant (Sowley *et al.*, 2010). There have also been reports of short phases of endophytic growth by *B. cinerea* within undamaged plant tissues for raspberry (Williamson *et al.*, 1987) blackcurrant (McNicol and Williamson, 1989) and strawberry (Bristow *et al.*, 1986).

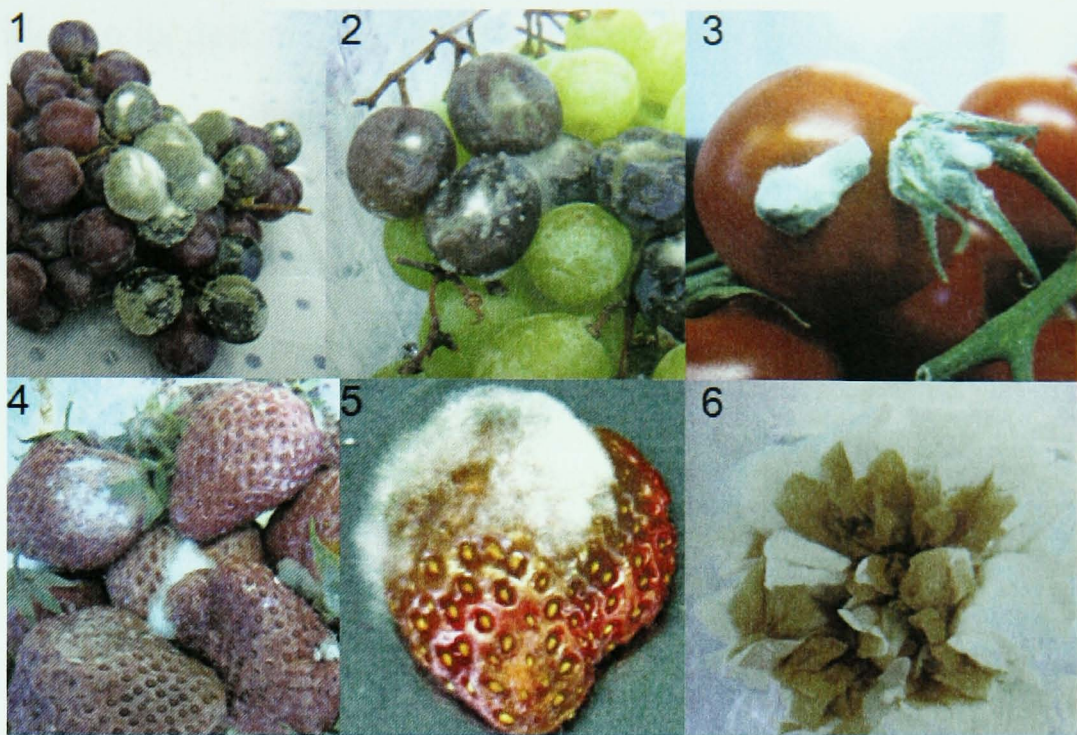


Figure 1.1 Typical symptoms of *B. cinerea* plates 1- 6 red grapes, white grapes, tomato, strawberries, strawberry, and rose respectively. Showing tissue softening and brown discolouration along with profuse aerial mycelia which eventually give rise to numerous dark gray conidia.

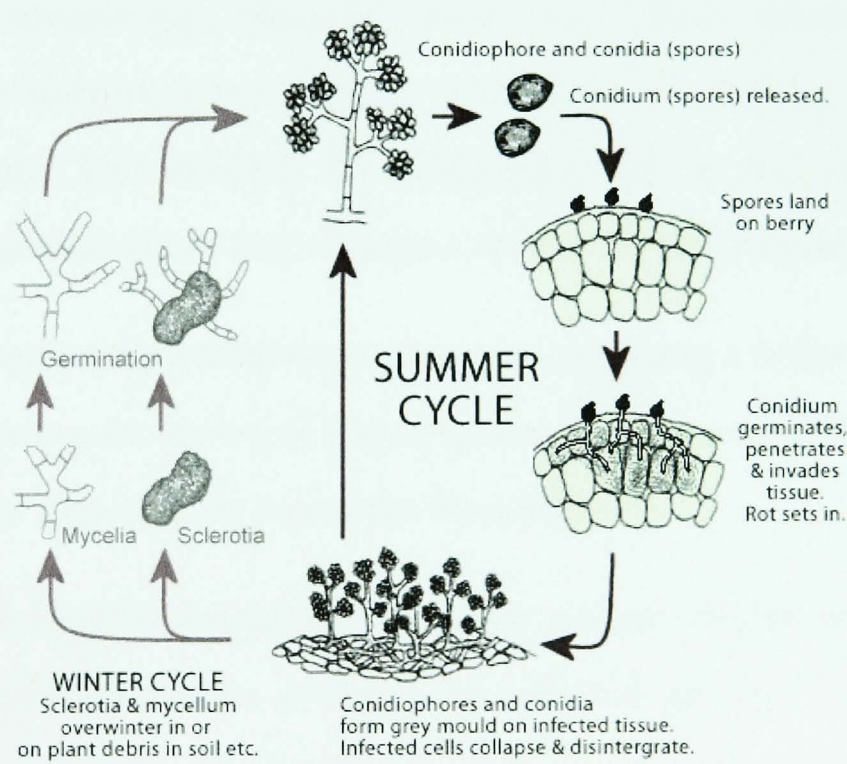


Figure 1.2 The asexual life cycle of *B. cinerea* starts as conidiophores producing conidia that spread by wind to susceptible host tissue. The summer cycle shows the continual production and dispersal of conidia and growth of mycelia. Unfavourable growing conditions such as the winter season induce the dormant period where the fungus overwinters either as mycelia or sclerotia (Fugelsang, 1997).

In addition to its detrimental impact as a serious plant pathogen, *B. cinerea* is important to some parts of the wine industry. Wine grapes deliberately infected with *B. cinerea* gives rise to the noble rot that produces internationally renowned botrytised wines that include Tokay from Hungary, Sauternes from France and Beerenauslese from Germany (MacNeil, 2000).

The possible origin of botrytised wine can be dated back to the Hungarian legends in 1630 (MacNeil, 2000). But a more popular story originated in Germany in 1775 where the producers at Schloss Johannisberg in the Rheingau region awaited harvest instructions of the estate owner, the Bishop of Fulda (MacNeil, 2000). The delivery of the instructions was unfortunately delayed allowing *Botrytis* to spread, causing loss to the harvest. The presumed worthless grapes were dispersed among the local community who produced a surprisingly pleasant sweet wine which became known as spatelese, late harvest wine. *Botrytis* contributed in making the wine by removing water and altering the polysaccharides in the grapes leaving behind a concentrated product of sugars, fruit acids and minerals.

Wine connoisseurs have described the wine as having a bitter finish on the palate and having an aroma of honeysuckle, and goes well with the strong flavour of blue vein cheeses especially Roquefort.

Winegrower's lung is an occupational lung disease caused when a person has inhaled *Botrytis* spores growing on infected grapes. The severity of symptoms depends on the length of exposure. Acute exposure results in symptoms such as shortness of breath, body aches, fevers and chills. Severe exposure results in lung scarring and respiratory failure. Exposure to *Botrytis* spores may also trigger hypersensitive pneumonitis for some individuals

with a history of ongoing medical conditions including asthma. Hypersensitive pneumonitis also known as extrinsic allergic alveolitis is interestingly protected against by smoking (Aresery and Lehrer, 2002).

Botryotinia fuckeliana, the teleomorph (sexual reproductive stage) of *B. cinerea* is heterothallic, having one locus mating type with two idiomorphs (MAT1-1, MAT1-2) (Faretra *et al.*, 1988; Coopin, *et al.*, 1997). Mating is initiated in favourable environmental conditions when uninucleate microconidia (antheridium) and multicellular sclerotia (ascogonium) release pheromone signals that allow recognition and fusion between each other to occur. The newly formed dikaryotic cells undergo meiosis and one round of mitosis producing asci with eight multinucleate ascospores (Lorenz and Eichorn, 1983). Apothecia which contain the asci protrude from the ascocarp fruiting body to expose the asci (Fig 1.3). Although production of apothecia is possible in the laboratory (Faretra and Antocacci, 1987), they are rarely identified in nature (Beever and Weeds, 2004). For asexual reproduction, conidia would usually have 3 - 6 similar nuclei (Grindle, 1979).

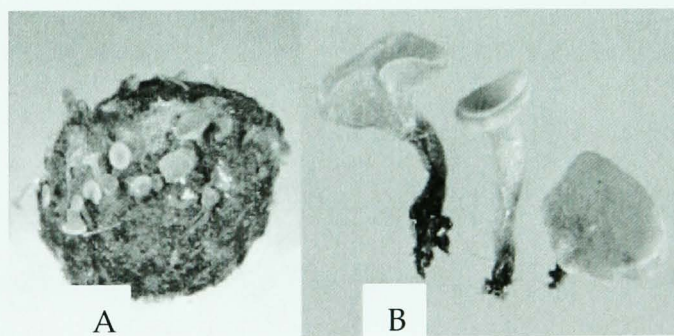


Figure 1.3 Apothecia of the teleomorph of *B. cinerea*, (A) showing apothecia growing from the ascocarp and (B) typical disk shaped formation of the apothecia (Beever and Weeds, 2007).

Fungi can share cytoplasmic and nuclear material by a process called anastomosis. This process occurs when hyphae from self or non-self origin (different genetic background) fuse to form a heterokaryon. If the fused fungi are compatible, the heterokaryon will develop and grow (Paoletti and Saupe, 2009). If there is vegetative incompatibility (VIC) between the fungal isolates, the heterokaryon will die. *Botrytis cinerea* has numerous vegetative compatibility groups (VCG) which include 59 VCGs identified in 82 strains from New Zealand (Beever and Weeds, 2004) and 15 VCGs in 21 strains from Israel and France (Korolev *et al.*, 2008). This shows that *B. cinerea* has a high level of VIC. The genes responsible for VIC in *B. cinerea* are not known (Glass *et al.*, 2000) but it may be possible that *B. cinerea* is similar to other *Ascomycete* fungi having 2 or more alleles for the numerous VIC genes, and that fusion would only occur when all the alleles are the same.

Most published genetic studies are on *B. cinerea* strains SAS405 and SAS56 (Beever and Weeds, 2004) with recent work including *Botrytis cinerea* B05-10 and T4 (ten Have *et al.*, 2009; Fischer *et al.*, 2009). This work uses *B. cinerea* B05-10 a strain treated with benomyl to reduce DNA content, which was said to originate from *B. cinerea* SAS56, but molecular analysis by Tudzynski and Siewers (2004) has disputed this. The fully sequenced and annotated genomes for *B. cinerea* B05-10 and T4 are available online at [http://www.broad.mit.edu/annotation/genome/botrytis_cinerea /Home.html](http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html) and [http://www.genoscope.cns.fr/spip/Botrytis-cinerea-whole-genome .html](http://www.genoscope.cns.fr/spip/Botrytis-cinerea-whole-genome.html).

1.2 Functional analysis of Dicer genes in *B. cinerea*

Gene knockout, also known as targeted gene disruption, is when a known gene in the organism's genome is removed and replaced by a selective

marker. The targeted gene is disrupted when there is homologous recombination between regions flanking the targeted gene and the gene disruption cassette carrying the selective marker. Gene disruption is used so that the phenotype of the desired mutants could be studied. This approach is used in this work and is explained in detail in the following Chapters.

The selective markers used in gene disruption are usually antibiotic and herbicide resistance genes that allow identification of successful transformants. In fungi, the selective markers used include the antibiotic resistance genes for phleomycin (Jain *et al.*, 1992), hygromycin (Leung *et al.*, 1989), nourseothricin (Kuck and Hoff, 2006) and the herbicide basta (Nayak *et al.*, 2005). These selective markers have been used in *B. cinerea* transformations (Tudzynski and Siewers, 2004).

To date, there are over 50 *B. cinerea* genes targeted by gene disruption, with about one third of the mutants reported to be altered in pathogenicity (Tudzynski and Siewers, 2004; Schamber *et al.*, 2009). Most of the genes in *B. cinerea* identified to be involved in pathogenicity were from the signalling pathways which included the mitogen activated protein kinases MAPK (*BMP1*), MAPKK (*Ste7*) and MAPKKK (*Ste11*) (Schamber *et al.*, 2009).

To learn more about the expression levels of a targeted gene, there are molecular techniques that can be used which include the use of non-invasive reporters. The reporters are genes that are fused to the regulatory region of the gene of interest (Cotlet *et al.*, 2001). When the promoter is active, the reporter will emit a light correlating to the level of gene expression. The reporters which have been successfully introduced into *B. cinerea* include the green fluorescent protein (GFP) and red fluorescent protein (dsRED) (Li *et al.*, 2007; Kamenidou *et al.*, 2006).

The GFP protein isolated from the *Aequorea victoria* jellyfish is made of 238 amino acids (26.9 kDa), and emits a green fluorescent colour when excited by blue light (Chalfie *et al.*, 1994). This protein has a major excitation peak of 395 nm and minor of 475 nm. Molecular improvements have been made to GFP producing the more photostable enhanced GFP and GFP mutants that emit a blue, cyan and yellow fluorescence when excited (Lippincott-Schwartz and Patterson, 2003).

The dsRED protein (28 kDa) isolated from a *Discosoma* genus of coral (Baird *et al.*, 2000) emits a red colour can be excited by a linear polarized light (Cotlet *et al.*, 2001). This protein has a major excitation peak of 490 nm and minor of 559 nm (Heikal *et al.*, 2000). Improvements to the maturation rate and increased fluorescence have produced the DsRED-2, DsRED-T1 and monomeric red fluorescence proteins (Bevis and Glic, 2002; Matz *et al.*, 1999).

Another way to determine the level of gene expression of the targeted gene is by Real-Time PCR (qPCR). This method identifies the accumulation of mRNA from the targeted gene in real-time using designed PCR probes and fluorescent dyes such as SYBR green (Higuchi *et al.*, 1992). In *Botrytis*, qPCR has been used to detect and quantify genes encoding the phytotoxic proteins BcNEP1 and BcNEP2, from the NEP1-like family (Arenas *et al.*, 2010). For this work, qPCR has been used to determine the expression levels for the targeted genes.

1.3 Control of *B. cinerea*

Botrytis cinerea can cause a lot of damage to crops, therefore several different control strategies have been employed to reduce its incidence and the damage it may cause. Most of the economic loss due to the pathogen is in the reduction in the quality of the crop rather than in the overall yield (Moss, 2008). However, these are usually high-value crops where visual appearance is very important to the consumer, which means that even low levels of infection may not be tolerated.

1.3.1 Cultural control

Crop management practices to reduce *B. cinerea* incidence should eliminate conditions that promote fungal growth. With crops grown in open canopy, there has to be sufficient air movement to lower the humidity and to prevent the accumulation of water droplets. For grapevines, the addition of summer pruning is very beneficial to increase air movement (Gubler *et al.*, 1987). It has been reported in the production of strawberries and raspberries that the use of plastic rain shelters and tunnels showed a reduction in *B. cinerea* occurrence by 90 %, probably due to reduced surface wetness and less rain splash (Xiao *et al.*, 2001). In greenhouses, good ventilation is important in reducing high relative humidity thereby minimizing wetting on the foliage (Williamson *et al.*, 2007). In cool green houses, heating before sunrise reduces dew formation on leaves and reduces incidence of *B. cinerea* (Dick and Wubben, 2004).

Adequate spacing between plants allows for light and air movement when the crops get larger. It is also recommended that growers manage insect

pests that would injure the plant as this would increase potential for infection and the pests may also act as vectors of disease (Fermaud and Gaunt, 1995). Another effective way of controlling *B. cinerea* is the continual removal of dead crop material and weeds that may produce inoculum.

Post harvest losses from *B. cinerea* are usually reduced by the use of cold chain marketing which deals with packaging, storage and distribution of produce at controlled temperatures of 4 °C (depending on the produce). This type of marketing increases the shelf life of produce and reduces *B. cinerea* occurrence and spread. The produce is carefully selected and prepared to protect against physical damage and minimises introduction of *B. cinerea* inoculum before entering the cold chain market (Kadar, 2005).

1.3.2 Natural resistance

Although there are a small number of breeding programmes trying to find *B. cinerea* resistant cultivars, no cultivar has been commercially used and no resistance genes have been mapped (Loebenstein *et al.*, 2009).

For plant resistance against *B. cinerea*, researchers have identified resistant cultivars of tomato (*Lycopersicum esculentum*) crossed with its wild relative (*Solanum lycopersicoides*) (Guimaraes *et al.*, 2004). The tests involved infecting detached leaves by droplet inoculation and also spray inoculation on entire seedlings. Resistance was demonstrated by hyphal lysis and death of conidia three days after inoculation (Guimaraes *et al.*, 2004). The mechanisms involved in the tomato resistance to *B. cinerea* have yet to be identified.

For some plant hosts, the mechanisms responsible for partial resistance to *B. cinerea* have been identified. Phenylpropanoid compounds and chitinases

produced by kiwifruit have been found to create resistance to *B. cinerea* by affecting conidial germination (Wurms *et al.*, 1999). For fungal resistance in *Arabidopsis* plants, the ethylene insensitive 2 gene (*ein2-1*) was important for resistance against *B. cinerea* since *ein2-1* mutants were more susceptible to fungal infection (Thomma *et al.*, 1999). Ethylene in tomato was found to be important for resistance to *B. cinerea*, since ethylene treated plants were less susceptible to the fungus (Diaz *et al.*, 2002). Although chemicals and gases have been found to be involved in resistance to the fungus, more work has to be done to understand the mechanisms involved.

In tomato plants, it was noticed that when researchers introduced a plasmid having a Cauliflower mosaic virus 35S promoter and an inhibitor of viral replication (IVR) cDNA, to reduce viral infection, resistance to *B. cinerea* also occurred (Loebenstein *et al.*, 2009). Fungal resistance was identified in less than 50 % of plants transformed, and the mechanism is unknown.

While work so far has focused mainly on resistance of some crops to *B. cinerea*, it must be considered that there remains a large number of other *Botrytis* species for future research. Also, research for host resistance has focused on just a few crops affected by *B. cinerea* and given the range of crops affected by *B. cinerea* means there is still a need for additional control measures. Although host resistance has potential to protect against *B. cinerea* and possibly increase produce yield, more research is required.

1.3.3 Chemical control

The use of chemical control is one of the primary ways to protect against *B. cinerea*, but this has some drawbacks. Several families of fungicides have been used to control *B. cinerea* such as benzimidazoles and dicarboximides (Vallejo, 2002). Benzimidazoles act by inhibiting β -tubulin polymerization while dicarboximides inhibit triglyceride biosynthesis. Some of the brands extensively used were Ornalin and Chipco 26019 for the benzimidazole based fungicides and Vinclozolin and Iprodione for dicarboximide based fungicides.

After successful applications of benzimidazoles and dicarboximides, resistant strains started to be reported (Bollen and Scholten, 1971; Hans-Juergen and Dietrich, 2000). In Europe, the control of *B. cinerea* was greatly hampered by fungicide-resistant strains (Stehmann and de Ward, 1996). The lack of alternative fungicides saw the continual use of dicarboximides and the considerable increase in resistant strains in the European regions in the 1980's (Leroux, 2004). Failure to control the fungus happened in several instances on a wide variety of strawberries, vegetables, greenhouse crops and vineyards (Lorenz, 1988; Katan, 1982).

The *B. cinerea* gene responsible for resistance to benzimidazole was *Mbc1* (Yourman *et al.*, 2000) while the *Daf1* gene was found to be involved dicarboximide resistance (Faretra and Pollastro, 1991). Discontinuation of fungicide application saw a general decline in frequency of dicarboximide-resistant isolates (Leroux, 1995).

Recently, new compounds that target *B. cinerea* have come to market include anilinopyrimidines, fludioxonil and the most promising fenhexamid.

The fungicide anilinopyrimidines prevents methionine biosynthesis in *B. cinerea* which inhibits mycelial growth, (Leroux, 1996; Rosslenbroich and Stuebler, 2000). The application of anilinopyrimidines on roses as vapours compared to droplets has been reported to be more effective in controlling *B. cinerea* (Grinstein *et al.*, 1997). This chemical, which acts as a contact fungicide, inhibits spore germination and mycelial growth by preventing methionine biosynthesis (Latorre *et al.*, 2002). Anilinopyrimidines have been effective in protecting apples and pears in the USA (Zhao *et al.*, 2009). The commercially available fenheximid fungicide, sold under the name Teldor is a sterol biosynthesis inhibitor (Leroux, 2004). The fungicide works by disrupting the sterol C-4 demethylation pathway (Debieu *et al.*, 2001). It has been reported that fenheximid was effective in controlling *B. cinerea* when applied during the flowering stages of grapevines (Petit *et al.*, 2010). Fenhexamid is the most reliable fungicide for the control of *B. cinerea* to date (Esteve-Turrillas *et al.*, 2010).

Though expensive, the use of chemical controls is effective, particularly when combined with sustainable and proficient pest management measures such as ventilation, hygiene and other pest control measures to ensure minimal chemical applications to reduce chemical residue and build up of fungal resistance.

1.3.4 Biological control agents

The use of biological control agents (BCA) to control *B. cinerea* is more appealing than chemicals since BCA are in most cases, safer for the environment and have less chance for the development of resistance (Son *et al.*, 2002). Despite such benefits however, the estimated world market for all

BCA is only £ 7 - 12 million sterling (Elad *et al.*, 2007), a tiny fraction of the global crop protection market which is some £ 10 - 15 billion sterling annually for fungicides alone.

Jacometi *et al.* (2010) gave a list of 21 commercially available BCA for the control of *B. cinerea*, which include the filamentous fungi *Trichoderma*, *Gliocladium* and *Ulocladium* that produce lysing enzymes. *Trichoderma* penetrate *B. cinerea* mycelia (Shtienberg and Elad, 1997), *Gliocladium* reduce pathogenicity (Paul, 1999) while *Ulocladium* partially hindered sclerotia production of *B. cinerea* (Dubos, 1992). Though this may give promising candidates for BCA for *B. cinerea*, the apparent success of the hypovirulent mycovirus in regulating the effect of *Cryphonectria parasitica* has created interest in using *B. cinerea* mycoviruses in an attempt to control the virulence of this devastating pathogen.

1.4 Mycoviruses

In 1962, experimental evidence demonstrated the existence of mycoviruses in diseased edible mushrooms, *Agaricus bisporus* (Ghabrial, 1980). One reason often suggested for the belated discovery of mycoviruses, compared to viruses of animals, plants and bacteria, is probably that most mycoviruses did not cause any readily detectable phenotypic changes in their hosts (Ihrmark, 2001). By 2009, over 80 mycoviruses have been identified (Pearson *et al.*, 2009).

The majority of mycoviruses discovered have RNA genomes rather than the more typical DNA genomes observed in animal viruses (Ball, 2007). There are more double stranded RNA mycoviruses than single stranded RNA

mycoviruses. Whilst the ssRNA viruses typically contain a single nucleic acid molecule, the dsRNA mycoviral genomes are usually segmented (Grogan *et al.*, 2003) and are often separately encapsidated into identical capsids (Ghabrial, 1980). Non-encapsidated mycoviral genomes reside as naked genetic elements (Grogan *et al.*, 2003). Mycoviruses are usually located in the cytoplasm of the fungal host, although some have been found in association with mitochondria (Van Diepeningen *et al.*, 2006).

1.4.1 Viral replication and movement

Viruses only replicate inside the living host using host derived machinery for much of the replication, meaning that their genomes are usually very small—often just a few genes (Smith and Helenius, 2004). When replication is initiated by the virus, proteins are made, which include the RNA-dependent RNA-polymerase (RdRp) and helicase (Tao and Ye, 2010) necessary for genome replication. For some plant viruses, the coat proteins and movement proteins may also be made to allow for encapsidation and movement through the host respectively (Gillespie *et al.*, 2002).

Most mycoviruses are apparently latent and replicate inside the fungus without showing any physiological changes on the host (Martin *et al.*, 2010). Some viruses alter the fungi's biological functions eventually killing the fungus while some cause the fungus to reduce severity of disease (decrease growth rate and sporulation). The latter has attracted interest due to its importance in agriculture and industry especially as biological controls.

1.4.2 Viral transmission

Mycoviruses survive in the host's cytoplasm and are transmitted when there is cytoplasmic exchange or formation of new cells. Mycoviruses are reliant on their fungal hosts for intracellular transmission and have no known extracellular mode of transmission (Buck, 1998). Whilst plant viruses have movement proteins which assist with movement between the hosts cells via plasmodesmata (Sasaki *et al.*, 2009), these have not yet been reported in mycoviruses.

The movement of mycoviruses in fungi of the same species is thought to be by hyphal anastomosis. Hyphal anastomosis is where two hyphal tips fuse and exchange genetic and cytoplasmic material. Since mycoviruses are present in the cytoplasm, they are included in the exchange (Hillman *et al.*, 1994). Vegetative incompatibility controls anastomosis (Glass *et al.*, 2000), but studies on *C. parasitica* have illustrated that viral exchange is possible between vegetative incompatible strains (Cortesi *et al.*, 2001). When two vegetative incompatible strains fuse, there is cell death, but when cell death is slow, there is a chance of genetic and cytoplasmic exchange, and hence viral transmission.

Viral transmission in fungi is also possible during ascospore formation, although it was reported that some mycoviruses could not be transmitted to newly formed ascospores (McFadden *et al.*, 1982; Coenen *et al.*, 1996). The mycoviruses present during fusion and meiosis of the sexual reproduction stage are transmitted to the new ascospore where the mycoviruses replicate in the new host. For fungi, the occurrence of mycoviral transmission through ascospores can range from 10 - 100 % (Van Diepeningen *et al.*, 2006; Chu *et al.*, 2004). Also, reports have identified similar mycoviruses in *Ascomycetes*

and *Basidiomyces* (Ikeda *et al.*, 2005) showing that viral transmission might not be limited to the same species, though the mechanism for this is not known (Pearson *et al.*, 2009).

1.4.3 Mycoviruses that alter fungal phenotypes

Whilst the role of viruses as pathogens in crop plants is well known, there is much less that is understood about the biology of mycoviruses. Mycoviruses discovered in the fungi which have received the most attention have been *A. bisporus* and *S. cerevisiae* due to their altered phenotypes and hence economic impact caused by the presence of mycoviruses.

Agaricus bisporus is seriously affected by several viral diseases, most notably La France disease and the Mushroom virus X (MVX) disease. La France disease which greatly affected commercial mushroom production was first reported in the USA in 1948 (Sinden and Hauser, 1950) and later in the UK (Ghabrial, 1994). Symptoms included no mycelial growth on mushroom beds, premature opening of mushrooms and malformed fruiting bodies (Romaine and Goodin, 2002). La France infected mushrooms were reported to contain an isometric viral particle with a diameter of 34 – 36 nm (Romaine and Schlagnhauer, 1995). This virion particle later named *A. bisporus* virus 1 (ABV1) (Van ver Lende *et al.*, 1996) was purified to show six dsRNA molecules: M2 (1.3 kb), L1 (3.8 kb), L2 (3.1 kb), L3 (3.0 kb), L4 (2.8 kb) and L5 (2.6 kb) (Harmsen *et al.*, 1989). The MVX disease was observed in several British farms in the mid 1990s. Mushrooms affected by the MVX disease showed arrested development of pins and brown discolouration. Twenty four dsRNAs have been identified as exclusively present in MVX infected

mushroom samples (Grogan *et al.*, 2003). There has yet to be a clear relationship between the MVX symptoms and the presence of any specific dsRNA elements (Grogan *et al.*, 2003).

The yeast *S. cerevisiae* used in baking and brewing have toxin producing strains. The presence of cytoplasmically inherited dsRNA viruses and toxin secreting proteins in *S. cerevisiae* started the research in yeast virology in the early 1960s (Bevan, 1963). It was known at the time that secreted protein toxins (Killer toxin) from certain strains (Killer yeasts) were lethal to sensitive strains. The killer groups of yeast consist of strains producing toxins encoded by cytoplasmic persisting dsRNA mycoviruses (Magliani *et al.*, 1997; Reiter *et al.*, 2005). These dsRNA mycoviruses presumably gave advantage to *S. cerevisiae* by restricting the multiplication of non-mycoviral strains which were not immune to the toxin. DsRNA mycovirus species in *S. cerevisiae* include the M and L species (Shmitt, 1990). The M species include the three major killer mycoviruses M-1, M-2 and M-28, each encoding a specific killer toxin K-1, K-2 and K-28 respectively, and specific immunity mechanisms (Scmitt, 2006). Maintenance of the M species relies on the helper virus L-A species by providing capsid and cap-pol. The killer toxins K-1 and K-2 disrupt cytoplasmic function while K28 has a unique and unusually stable toxin that blocks DNA synthesis after entering the target cell by receptor-mediated endocytosis resulting in capase-mediated apoptosis (Breinig *et al.*, 2006). Immunity to the toxin is possible by degradation of the toxin and precursor molecule complexes (Breinig *et al.*, 2006).

1.4.4 Mycoviruses associated with biological control

Chestnut blight caused by *C. parasitica* originated from Japan where it was a minor pathogen of local trees (Yir-Chung, 2007). Within increased international trade in timber, the fungus managed to arrive in United States of America in the early 1900's (Milgroom and Cortesi, 2004) and within 40 years it devastated the American chestnut forests (Perlerou and Diamandis, 2006). In Europe, the disease was first found in Italy in 1938 and later in 1947 and 1956 in Spain and France respectively, and is still present in chestnut cultivations to date (Perlerou and Diamandis, 2006). After the initial destruction of forests in the European countries, evidence was seen of the decline in fungal virulence and recovery for the chestnut trees. It was found that *C. parasitica* mycoviruses induced hypovirulence in the fungus allowing the chestnut trees to recover from fungal infection by creating a callus over the canker lesion (Ding *et al.*, 2006). This hypovirulent trait has been exploited to allow biological control of the pathogen in the USA, where deliberate introduction of virally infected strains allowed the virus to spread through the fungal population, thereby decreasing virulence and reducing the damage to chestnut forests. The success of this viral-based biological control raises the possibility that it may be applicable to other fungal plant pathogens such as the important gray mould causing fungus, *B. cinerea*.

Many different hypovirulent mycoviruses have been identified for *C. parasitica* (Linder-Basso *et al.*, 2005). Most of these viruses are single-stranded 'positive' RNA from the Hypoviridae family which has four species namely CHV1, CHV2, CHV3 and CHV4 (Linder-Basso *et al.*, 2005). These hypovirulent mycoviruses also suppress pigmentation, reduce asexual reproduction and cause loss of female fertility in *C. parasitica* (Lin *et al.*, 2007).

Upon infection, CHV-1 down regulates transcription of several genes in *C. parasitica*, including the sex pheromone genes (*Vir1* and *Vir2*), a gene encoding a cell wall hydrophobin (*Crp*) and a gene encoding an extracellular laccase (*Lac1*) (Kazmierczak *et al.*, 1995). The mRNA expression levels for these genes in the CHV1 infected strain (UEP1) compared to a virus free strain (EP155/2) showed *Vir1* and *Vir2* being undetectable and *Crp* and *Lac1* being reduced by 50 – 70 % (Kim *et al.*, 1995; Kazmierczak *et al.*, 1995), but the importance of these events in hypovirulence is not clear.

1.4.5 *B. cinerea* mycoviruses

Several studies on *B. cinerea* have investigated the incidence of mycoviruses (Castro *et al.*, 1999; Vilches and Castillo, 1997; Howitt *et al.*, 2001; Howitt *et al.*, 2006). Most of the data on mycoviruses so far are focused on a rather limited collection of *B. cinerea* isolates and are often from a single locality or from a single host plant.

In *B. cinerea* strain 55 k isolated from grapes (Germany), a 28 nm dsRNA mycovirus with a 1.8 kb genome segment encapsidated within a isometric protein coat had been identified (Castro *et al.*, 1999). The mycoviruses main structural component was a 68 kDa polypeptide. Another *B. cinerea* strain, CVg25 isolated from grapes (Chile), had mycoviruses with three dsRNA having molecular sizes of 8.3 kb (L), 2.0 kb (M1) and 1.4 kb (M2) (Vilches and Castillo, 1997). *B. cinerea* cells that were infected with these mycoviruses showed evidence of cellular degeneration.

Botrytis cinerea strain CCg425 isolated from grapes (Chile) was found to have a 6.8 kb dsRNA mycovirus (Castro *et al.*, 2003). Virulence bioassays on bean

leaves showed that *B. cinerea* strain CCg425 was less aggressive than the virulent *B. cinerea* strain CKg54 which is not infected with dsRNA mycoviruses. Viral particles purified from hypovirulent *B. cinerea* CCg425 were transfected into the virulent *B. cinerea* CKg54 resulting in diminished virulence of the virus infected fungus. The *B. cinerea* CCg425 strain illustrates that the dsRNA mycovirus associated with the strain was able to confer hypovirulent-associated traits to its host.

The mitovirus BcMV1 which was isolated from *B. cinerea* found on *Brassica napus* (China) was reported to confer hypovirulence to *B. cinerea* CanBc-1 (Zhang *et al.*, 2010). The production of typical *Botrytis* infection cushions were formed on onion bulbs when infected with the virulent *B. cinerea* CanBc-1c-66 and *B. cinerea* CanBc-2 strains, but infection cushions were not formed and reduction in mycelial growth was shown when infected with *B. cinerea* CanBc-1. This shows that the mitovirus conferred hypovirulence traits to *B. cinerea* CanBc-1.

In a larger study, two hundred *B. cinerea* isolates from a range of hosts were analysed for dsRNA elements in New Zealand and revealed that 72 % of the strains contained dsRNA with high variation in dsRNA profiles, between and within groups of fungal isolates from different hosts (Howitt, 1998). Also, flexuous rod shaped elements were found in isolates that lacked obvious dsRNA elements (Fig 1.4). Further study at the molecular level gave the first complete nucleotide sequences and molecular characterization of two *B. cinerea* single stranded RNA (ssRNA) mycoviruses named *Botrytis* virus F (BVF) and *Botrytis* virus X (BVX) isolated from *B. cinerea* RH106-10 (Howitt *et al.*, 2001; Howitt *et al.*, 2006).

BVF contains a 6.8 kb ssRNA genome with a poly (A) tract at the 3' end. Analysis of genomic cDNA sequences showed two open reading frames (Fig 1.5 A). ORF1 had a protein sequence similarity to the RdRp of plant 'tymo-' and 'potex-like' viruses and methyltransferase similarity to 'tymo-like' viruses. It also contained an opal putative readthrough codon between the helicase and the RdRp region; a feature not reported for 'tymo-' and 'potex-like' replicase sequences. ORF2 shared protein sequence similarity with protein coats to those of 'potex-like' viruses.

Mycovirus BVX was the second ssRNA viral genome from *B. cinerea* to be sequenced and characterised (Fig 1.5 B) (Howitt *et al.*, 2006). BVX contained a 6.9 kb genome and had a poly (A) tract at the 3' end. Analysis of the genomic cDNA sequence revealed 5 ORFs. ORF1 showed protein sequence similarity to replicase proteins of plant 'potex-like' viruses. ORF 3 shared protein sequence homology to coat proteins of plant 'potex-like' viruses. The other ORFs showed no significant sequence homology to any known protein sequence.

The ssRNA mycoviruses are able to replicate in the host using their own RNA dependent RNA polymerase (Waterhouse *et al.*, 2001). During replication there is a dsRNA intermediate stage where complementary strands are synthesized as templates to make more mycoviral genomes.

Unlike most plant viruses that have a movement protein to allow translocation with the host, some mycoviruses, including BVF and BVX do not require the movement protein since the fungal host has pores in the septa that allows for movement of fungal organelles and so, presumably, also mycoviruses.

Mycoviruses BVF and BVX were the chosen for this study because they are fully sequenced, were available in our group and have monopartite positive-sense ssRNA genomes making manipulation more amenable, unlike other mycoviruses that have multipartite or dsRNA genomes.

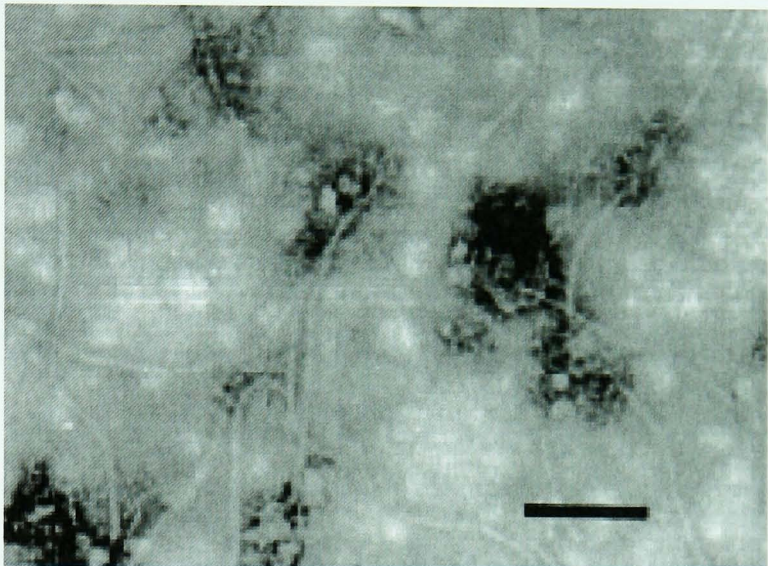


Figure 1.4 Electron micrograph of flexuous rod-shaped particles. The flexuous rod-shaped particles were partially purified from *B. cinerea* isolate RH106-10 and negatively stained with 2 % potassium phosphotungstate, Bar, 200 nm. (Howitt *et al.*, 2001).

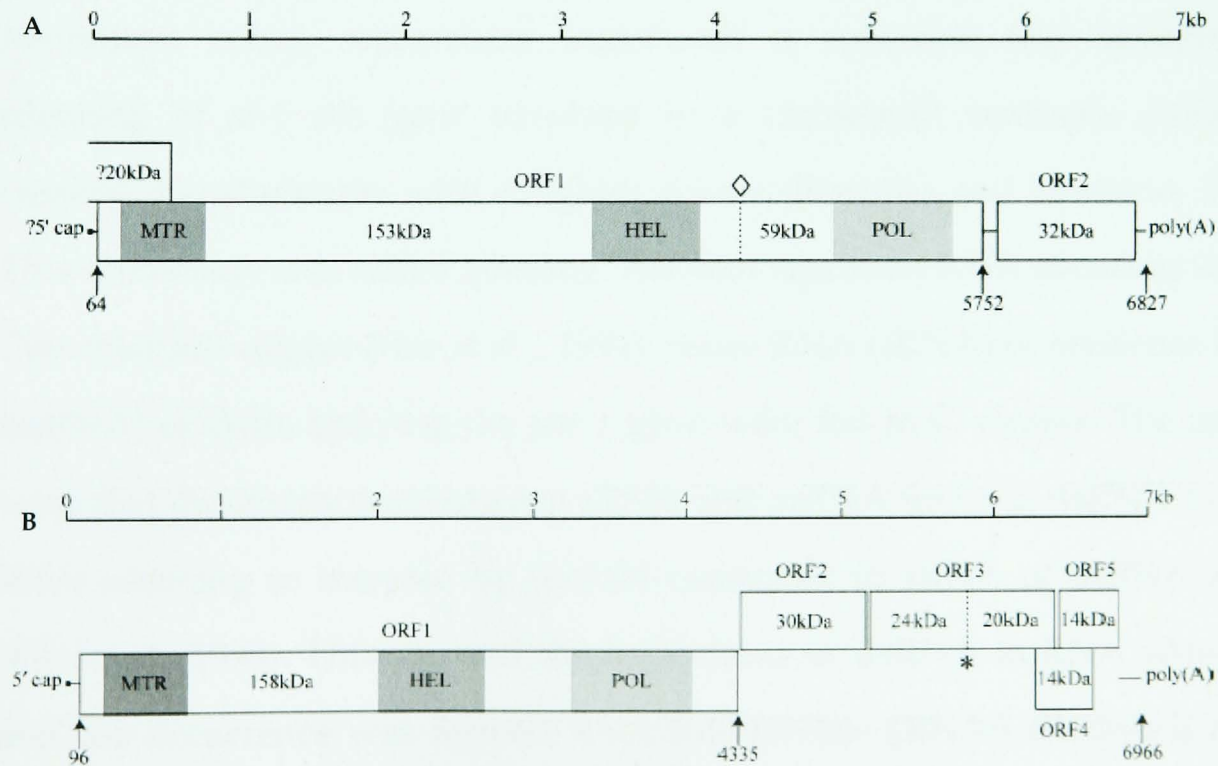


Figure 1.5 Schematic representation of the genome organization for *B. cinerea* mycovirus BVF and BVX: A: BVF with the two open reading frames which are boxed, diamond indicates position of the putative readthrough codon. B: BVX with five open reading frames which are boxed. Shaded boxes indicate relative positions of the conserved domains of the replicase gene (Howitt *et al.*, 2001).

1.5 RNA silencing

The history of RNA silencing starts with the suppression of β -galactosidase mRNA induced by constructs expressing the complimentary RNA strand in *Escherichia coli*. Up to 98 % inhibition of β -galactosidase protein synthesis was reported when using the β -galactosidase antisense mRNA (Pestka *et al.*, 1984). For petunia flowers, RNA silencing was first noticed when researchers introduced the *chalcone synthase* transgene (CHS) into the plants. The result was the suppression of CHS and the flowers becoming variegated or white (Napoli *et al.*, 1990). This occurrence was named co-suppression because of inhibition of both the homologous endogenous gene and the transgene. In *Neurospora crassa*, researchers introduced a construct that resulted in silencing of *al-1* the gene involved in a carotenoid synthesis pathway, creating transformants with a lighter colour (Romano and Machino, 1992). This occurrence was called quelling. Another report on RNA silencing was in *Caenorhabditis elegans* (Fire *et al.* , 1991). Sense RNA (sRNA) or antisense RNA (asRNA) or both, carrying the *par-1* gene were fed to *C. elegans*. The results were that the interaction between sRNA and asRNA forming dsRNA caused RNA silencing to increase by tenfold compared to sRNA or asRNA alone (Fire *et al.*, 1998). This showed the importance of dsRNA in RNA silencing, and the occurrence was termed RNA interference (RNAi) (Kadotani *et al.*,

2004). Since there are many terms for this occurrence, I will refer to it by its generically preferred name 'RNA silencing'.

RNA silencing is usually triggered by the detection of dsRNA molecules – which are not normal constituents of the cytoplasm. For plants, if the exogenous molecule is positive-sense single-stranded RNA (ssRNA) it is first converted to double-stranded RNA (dsRNA) by RNA-dependent RNA-polymerases (RdRp) to initiate RNA silencing (Fig 1.6). The dsRNA molecules of either exogenous or endogenous origin are recognized by the DICER protein and cleaved into short interfering RNA (siRNA) and microRNA (miRNA) of 21-25 bp in size (Baulcombe, 2002). The short duplexes are separated and one strand is used by the RNA induced silencing complex (RISC) as a guide strand (Vance and Vaucheret, 2001). RISC uses the guide strands to identify and pair with any RNA molecule with complimentary sequence, and then induces their cleavage using the catalytic endonuclease component of RISC; the argonats. When activated, this silencing system has the potential to reduce the titre or even eliminate viral RNA elements from the cytoplasm, and so could impact on the ability of a virus to replicate and cause disease.

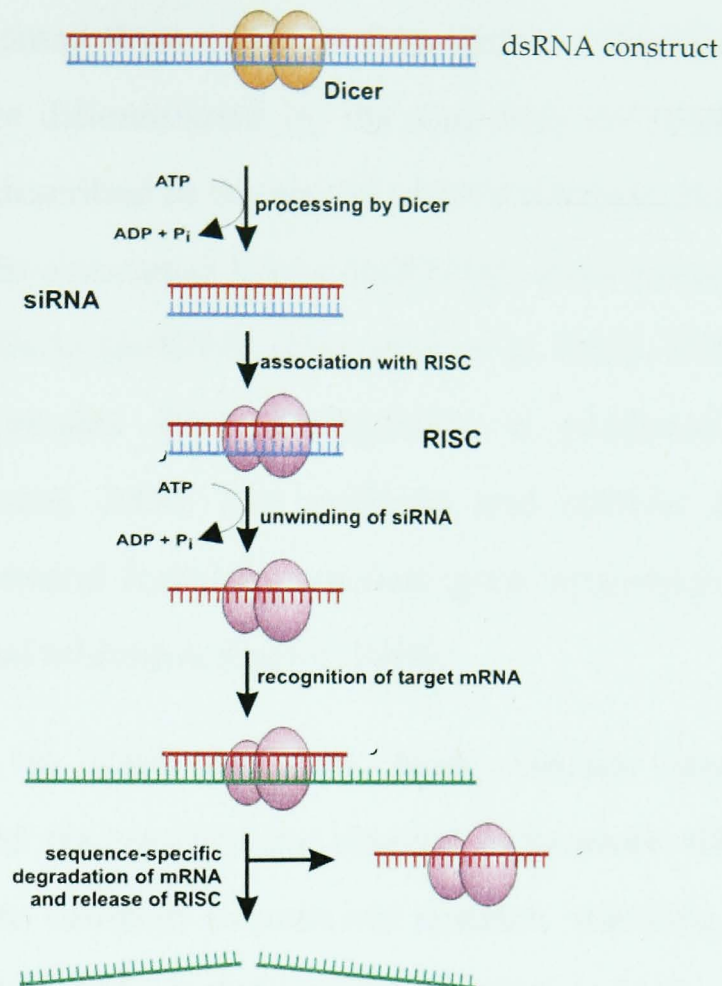


Figure 1.6 Illustrations for RNA silencing: In the cytoplasm, RNA silencing is initiated by dsRNA constructs (illustrated) or ssRNA strands that are recognised by RNA dependent RNA polymerases (RdRp) and creates complementary strands making dsRNA. The DICER protein recognises the dsRNA and cleaves them into ca. 21 bp fragments. The fragments are separated and one of the strands is used by the RNA induced silencing complex (RISC) as a template to recognise and degrade aberrant mRNA (from Rutz and Scheffold, 2004).

1.5.1 DICER protein

The DICER protein is an evolutionary conserved RNase III enzyme (Bernstein *et al.*, 2001) which initiates RNA silencing by recognizing and cleaving dsRNA into 21 – 25 bp siRNA, with 2 bp overhangs at the 3' end (Collins and Cheng, 2005). DICER also helps load the siRNA into RISC which directs gene silencing by targeted mRNA degradation (Bernstein *et al.*, 2001).

Of the many different small RNA duplexes (Vaucheret, 2006), there are three main classes which are differentiated by the way they are made and their molecular features as described in the model plant *Arabidopsis thaliana*. There are the heterochromatin-associated RNAs (hcRNAs), short interfering RNAs (siRNAs) and micro RNAs (miRNA) (Havecker *et al*, 2010). HcRNA which directs asymmetric cytosine DNA methylation is produced by RdRp (Broadersen and Voinnet, 2006). The miRNAs and siRNAs which have interchangeable biochemical functions regulate gene expression by mRNA cleavage or translational inhibition (Bartel, 2004).

DsRNA, synthesized *in vitro* and *vivo* from viruses, transgenes or endogenously activated transposons are cleaved to produce siRNA. These siRNA are proposed to function in antiviral defence, silencing aborted or overproduced mRNAs and guarding the genome from transposon disruption (Mello and Conte, 2004; Valencia-Sanchez *et al.*, 2006).

The siRNA can fall into two classes being either thermodynamically symmetric or asymmetric after being unwound (Guiliang Tang, 2005). Symmetric siRNA have equally stable ends making incorporation of both strands into the RISC complex equally efficient. On the other hand, asymmetrical siRNA only have one stable end, making unwinding easier from the less stable end, allowing only one strand preferable for incorporation into the RISC complex.

The miRNA are produced from natural dsRNA hairpins. For miRNAs, most of the strands are asymmetrical allowing efficient asymmetrical assembly of miRNA into RISC (Schwarz, 2003).

1.5.2 The role of the RNA silencing Dicer protein in fungi and plants

Studies have determined that a major role of the RNA silencing mechanism is the protection of the host against RNA elements, both exogenous (e.g. viruses and transposable elements) and endogenous (e.g. *bcsod1*) (Patel *et al.*, 2008). It is not present in all species for example the RNA silencing machinery does not exist in *Saccharomyces cerevisiae* and *Ustilago maydis* (Aravind *et al.*, 2000; Kamper *et al.*, 2006).

Fungi were reported not to have miRNAs (Murphy *et al.*, 2008), however Lee *et al.*, (2010) recently published findings in *N. crassa*, where one of the ARGONAUT proteins (QDE-2) was reported to be involved in the production of miRNAs, suggesting these are present in fungi.

Figure 1-7 illustrates the conserved domains for a typical fungal DICER protein in the *Ascomycota* phylum using the DICER protein sequences from *N. crassa*, a species with 2 Dicer-like genes (<http://www.ncbi.nlm.nih.gov/>). A typical DICER protein would contain a Dead box which is involved in structural alterations to the RNA molecule (Rocak and Linder, 2004), a helicase to assist incorporation of the guide strand into RISC (Soifer *et al.*, 2008), a duf283 domain of unknown function (Kadotani *et al.*, 2004), two RNA cleaving RNases III motifs (MacRae, 2007) and a double stranded RNA binding domain (dsRBD; Provost *et al.*, 2002).

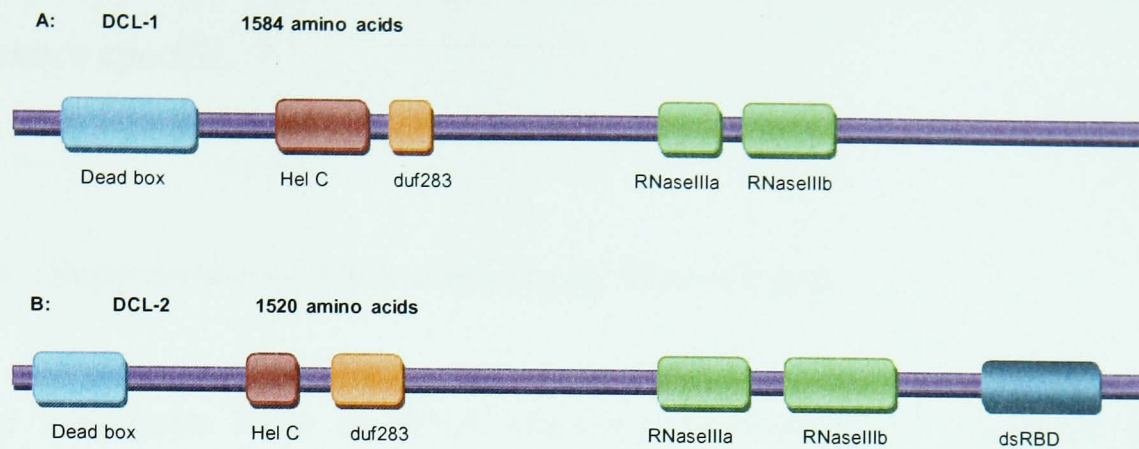


Figure 1-7 Illustration showing the probable arrangement of the DICER domains for *dcl-1* and *dcl-2* *N. crassa* (adapted from NCBI).

1.5.3 Argonauts

The ARGONAUTS and their related proteins are effectors of the RNA silencing mechanism being the key catalytic part of the RISC complex (Havecker *et al.*, 2010). There are three main groups of ARGONAUT proteins in *Eukaryotes* classified as ARGONAUTS, ARGONAUT-like or PIWI (P-element induced wimpy testis) according to the sequence of their PIWI and PAZ (PIWI Argonaut Zwillie) domains (Havecker *et al.*, 2010). The PIWI domain binds to the 5' end of the siRNA and cleaves targeted RNA for most family members (Steiner and Plasterk, 2006). The number of Argonaut genes varies between organisms, though most *Eukaryotes* have at least one; *Schizosaccharomyces pombe* and *Arabidopsis* both have one ARGONAUT, *Drosophila* has two, *B. cinerea* has four and humans have eight (Verdel *et al.*, 2004; Vaucheret *et al.*, 2004).

The ARGONAUT cleavage mechanism known as slicer is dependent on the ARGONAUT proteins and RNase-H like structure (Liu *et al.*, 2004). A feature commonly seen with ARGONAUT proteins studied so far is their association

with small RNA guides (siRNA and miRNA) making RNA silencing sequence specific.

1.5.4 Suppression of RNA silencing by Mycoviruses

Most *Eukaryotes* have an RNA silencing mechanism to inactivate highly expressed RNAs in the cytoplasm in a sequence specific manner (Chapman *et al.*, 2004). RNA silencing is explained later in this chapter but it is often active against RNA viruses. In plants, some viruses have adapted ways to suppress the RNA silencing mechanism. For the Tobacco etch potyvirus, it encodes a viral suppressor of silencing at the 5' proximal region of its genome termed the P1/HC-pro sequence (Koonin and Dolja, 1993; Endres *et al.*, 2010). P1 is involved in RNA replication while HC-Pro is involved in viral transport (Verchot and Carrington, 1995; Klein *et al.*, 1994) and suppression of RNA silencing (Pruss *et al.*, 1997). The Turnip mosaic virus contains a P1/HC-pro component which suppresses the RNA silencing mechanism in *Arabidopsis*, by interfering with miR171 activity (Kasschau *et al.*, 2003). It has been reported that the P1/HC-pro component targets suppression of the hosts RNA silencing mechanism at the RISC assembly and possibly unwinds the miRNA duplexes (Chapman *et al.*, 2004).

Another RNA silencing suppressor is the 2b protein which is encoded in Cucumber mosaic virus (CMV) sequence (Gonzalez *et al.*, 2009). The 2b protein carried by a vector was able to be active in suppressing RNA silencing when transformed into *Nicotiana benthamiana* and other *Solanaceous* plants (Brigneti *et al.*, 1998). The 2b protein localises to the nucleus by using a karyopherin α -mediated system (Wang *et al.*, 2004). The 2b protein targets

the RNA silencing mechanism for suppression by binding to the siRNAs, interfering with RISC activity (Goto *et al.*, 2007).

The two suppressers P1/HC Pro and the 2b proteins target the RNA silencing mechanism differently. P1/HC Pro suppressed silencing in tissues where silencing had already been initiated while the 2b proteins affected the initiation of silencing (Beclin *et al.*, 1998). Other viruses involved in the suppression of RNA silencing have been the potex-, tobamo- and geminiviruses which are targets and activators of RNA silencing (Beclin *et al.*, 1998).

The suppression of the RNA silencing mechanism is a common approach among plant viruses (Voinnet *et al.*, 1999). Strongly suppressive or evasive viruses have more potential to be highly pathogenic while those susceptible to silencing are more likely to be non-pathogenic or weak. The interactions between the virus and host could suggest strong selective pressures for the virus to have suppressors for RNA silencing, and it is possible that such mechanisms may be present in mycoviruses to evade fungal defence pathways.

1.5.5 RNA silencing vectors and their application

RNA silencing causes a reduction in gene expression, known as gene knockdown, which provides an effective and convenient tool for determining gene function (Nakayashiki and Nguyen, 2008).

The use of sense and antisense constructs to induce RNA silencing has been reported in *B. cinerea* (Patel *et al.*, 2008). Although sense and antisense constructs were able to induce silencing of *B. cinerea* superoxide dismutase

(SOD) transcripts, the antisense construct showed a higher level of silencing for the transformants on selection media.

The most reliable strategy to induce RNA silencing in filamentous fungi has been the use of a hairpin RNA (hpRNA) expressing plasmid (Goldoni *et al.*, 2004). Originally, the plasmids were created with inverted repeats, but this created problems since *E. coli* strains failed to propagate the plasmids. This was overcome with the introduction of spacer sequences between the two differently orientated DNA regions (Das Gupta *et al.*, 1987; Lobachev *et al.*, 1998). The spacer sequences of 500 -1022 bp resulted in efficient silencing (De Buck *et al.*, 2001). HpRNA plasmids were found to be reliable and efficient in the *Basidiomycete* yeast *Cryptococcus neoformans* (Liu *et al.*, 2002). The hpRNA plasmids have been constructed having PCR based inserts from several different vectors. The pSilent-1 vector was constructed for *Ascomycete* fungi (Nakayashiki *et al.*, 2005). The high throughput vector pTroja was the pSilent-1 vector adapted with Gateway technology (Shafran *et al.*, 2008). The pFANTAi4 uses Gateway technology and a GFP sentinel system (Krajaejun *et al.*, 2007). Another is the pSUPER RNAi system which uses the Human polymerase III RNA promoter (Brummelkamp, *et al.*, 2002) to direct transcription of the insert. This system was able to silence GFP expression in *Coprinus cinerea* (Costa *et al.*, 2008). These plasmids are important in RNA silencing but are generally not applicable with moderate scale analysis because these vectors normally require a two step orientated cloning (Nakayashiki and Nguyen, 2008). This problem is avoided using RNA silencing constructs that require one-step for construction. The opposing-dual promoter system has sense and antisense RNase II promoters that could have the target gene sense and antisense RNA transcribed independently.

This vector has been developed for *Histoplasma capsulatum* and *M. oryzae* (Rappleye *et al.*, 2004; Nguyen *et al.*, 2008).

Although there are proven RNA silencing systems available for use in fungi, the pLOB1 vector system previously used in *B. cinerea* was used for this work (Patel *et al.*, 2010). The pLOB1 vector system has been reported to silence the endogenous *B. cinerea* argininosuccinate synthase gene (*bcass1*). The use of this system meant less time was used for construction of a new vector. More on this system is detailed in the following chapters.

It has been seen from studies on other fungi that the DICER protein in the RNA silencing pathway acts to recognise invading RNA viruses and initiate the RNA silencing pathway (Segers *et al.*, 2007). Work on *B. cinerea* has shown that RNA silencing exists in this species as previously reported (Patel *et al.*, 2008). Since the importance of the DICER proteins in RNA silencing is now known from other organisms, it would be of interest to understand the role of the DICER proteins in *B. cinerea*. To date, no material has been published on *B. cinerea* *Dcr1* and *Dcr2*. So, to understand RNA silencing in *B. cinerea* especially the role the Dicer genes, the following objectives were outlined;

- Create *B. cinerea* Dicer disruption mutants for both genes. Experimentation would include the designing and construction of Dicer gene disruption plasmids, which would be transformed into *B. cinerea*. Recovered Dicer mutants would be observed for possible morphological changes compared to *B. cinerea* wild type
- Introduce an arginine silencing inducing construct into the *B. cinerea* Dicer mutants and wild type to elucidate the possible roles for the Dicer genes. If RNA silencing is identified in the wild type but not in

mutants, this would suggest the Dicer genes are involved in RNA silencing.

Another part of the study was to determine the relative expression levels for the putative RNA silencing genes in selected *B. cinerea* lines.

The final part of the study was to understand the interactions between *B. cinerea* and *Botrytis* mycoviruses and, to determine whether BVF has a potential as a biological control agent. This would include the transfection of the fully sequenced ssRNA mycovirus, *Botrytis* virus F (BVF), into mycovirus free strains of *B. cinerea*. The objectives were;

- Transfect mycovirus free strains with BVF by anastomosis and protoplast fusion. Anastomosis is the natural way mycoviruses are transferred between fungal hosts having vegetative compatibility. Protoplast fusion is the introduction by removing the cell wall increasing the probability of mycoviruses transfer between hosts.
- Analyse the transfected lines by molecular analysis to determine whether the presence of BVF has any effect on the new hosts. This would be determined by analysing mycoviral titre levels and gene expression levels for selected genes using qPCR.
- Determine the radial growth rates between the transfected lines and controls on growth media and selected fruits and vegetables to show if the presence of BVF has any effect on virulence.

Together these aims would give a better understanding of the genes involved in RNA silencing in *B. cinerea* and the relation between *B. cinerea* and its mycoviruses.

2 Materials and Methods

2.1 Strains

2.1.1 Fungal strains

Fungal strains used were *B. cinerea* haploid strain BO5-10 (Germany) (Buttner, 1994) which were obtained after benomyl treatment of strain SAS56, a viti-field isolate (Italy) (van der Vlugt-Bergmans, 1993). *B. cinerea* ku70 (Choquer *et al.*, 2008) which lacks the ku70 gene was derived from *B. cinerea* B05-10. The mycoviral containing *B. cinerea* strains REB171-3, RH205-10 were isolated from grapes fields while RE106-10 and RE105-19 were isolated from strawberry fields (New Zealand) (Howitt *et al.*, 1995).

2.1.2 Yeast strains

Saccharomyces cerevisiae strain BY4742 (acc # Y10000) (*MAT α* , *his3 Δ 1*, *leu2 Δ 0*, *lys2 Δ 0*, *ura3 Δ 0*) (Brachmann *et al.*, 1998) was obtained from Euroscarf Institute of Microbiology, University of Frankfurt, Germany.

2.1.3 Bacterial strains

Bacterial cells used for transformation were One shot® TOP 10 Chemically Competent *E. coli* [genotype: F-*mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*araleu*) 7697 *galU* *galK* *rpsL* (StrR) *endA1* *nupG*] and MAX Efficiency®. DH5 α ™ Competent cells, [genotype: F- ϕ 80*lacZ* Δ M15 Δ

(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (*r_k⁻*, *m_k⁺*) *phoA supE44 λ-thi-1 gyrA96 relA1*], (Invitrogen Corp., UK).

2.2 Media

2.2.1 Fungal media

Malt extract agar [MEA: malt extract 45 g l⁻¹ (Fluka, Fisher scientific, UK), mycological peptone 5 g l⁻¹ (Oxoid, UK), agar 18 g l⁻¹] and malt extract liquid media (ME: malt extract 10 g l⁻¹) was used to grow *B. cinerea*. SH agar (SHA: 0.6 M sucrose, 5 mM HEPES, 1mM HEPES, 1 mM NH₄H₂PO₄ and agar 1.2 % w/v) (Hamada *et al.*, 1994) was used for protoplast regeneration. Gamborg media [GM: Gamborg B5 3.16 g l⁻¹ (DUCHEFA Biochemie B.V., Netherlands), NH₄H₂PO₄ 1.1 g l⁻¹, glucose 10 g l⁻¹, agar 18 g l⁻¹ and +/- L-arginine 0.2 g l⁻¹] was used for arginine selection to identify the arginine silencing effect shown by reduced fungal lateral hyphal growth compared to wild type.

2.2.2 Yeast media

YPD liquid media [yeast extract 10 g l⁻¹ (Formedium, Switzerland), peptone 20 g l⁻¹, D-glucose 20 g l⁻¹] and YPD agar (YPD with agar 20 g l⁻¹) was used for yeast growth. Yeast synthetic dropout media, [YSDOM: yeast nitrogen base without amino acids 1.7 g l⁻¹ (Fluka, Fisher scientific, UK), casein hydrolysate 5 g l⁻¹, ammonium sulphate 5 g l⁻¹, adenine 20 mg l⁻¹, tryptophan 20 mg l⁻¹ and D-glucose 10 g l⁻¹] and YSDOM agar (YSDOM with agar 20 g l⁻¹) was used as selection media.

2.2.3 Bacterial media

For bacterial growth, Luria Bertani liquid media [LB, tryptone 10 g l⁻¹ (Melford, UK), yeast extract 5 g l⁻¹, NaCl 10 g l⁻¹], Luria Bertani agar (LBA, LB and agar 18 g l⁻¹), super optimal broth (SOB) (Tryptone 20 g l⁻¹, yeast extract 5 g l⁻¹, NaCl 10 g l⁻¹, KCl 2.5 g l⁻¹, MgCl₂ 10 g l⁻¹, MgSO₄ 10 g l⁻¹) and super optimal broth with catabolite repression (SOC; tryptone 20 g l⁻¹, yeast extract 5 g l⁻¹, 10 mM NaCl, 4 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) were used.

2.2.4 Chemically competent *Escherichia coli*

Chemically competent cells were prepared as described by Ausubel *et al* (2010). Five ml LB liquid media was inoculated with *E. coli* DH5 α ™ from glycerol stocks and incubated, shaking overnight at 37 °C, 250 μ l was used to inoculate 62.5 ml SOB and incubated, shaking at 37 °C until the optical density reached 600 nm (*ca.* 4 h), centrifuged at 460 g for 5 min at 4 °C and the pellet resuspended in 20 ml TB (0.25 M PIPES, 1 M KCl pH 6.7), chilled on ice for 10 min, centrifuged at 460 g for 5 min at 4 °C, resuspended in 5 ml TB, chilled on ice for 10 min, then aliquoted and stored ready for use in -80 °C.

2.3 Growth conditions

2.3.1 *Botrytis* growth condition

To grow *B. cinerea* for mycelia or conidia, MEA plates inoculated with *B. cinerea* conidia were incubated at 25 °C for about 7 days. For DNA and RNA extraction, mycelia was harvested by adding 10 ml 1 % Tween -80, scraping to loosen, and collected mycelia incubated at 25 °C shaking at 30 g in 50 ml ME liquid media for 18 h. For transformations, conidia was harvested by adding 10 ml 1 % Tween -80, scraping to release conidia and filtered through a 70 µm filter to remove mycelial fragments. Approximately 3×10^6 spores were used to inoculate 50 ml ME liquid media and incubated at 25 °C shaking at 3 g for 18 h.

2.3.2 Yeast growth conditions

A single yeast colony from yeast streaked on YPD plates was used to inoculate a 3 ml YPD liquid media and incubated at 30 °C overnight, shaking at 20 g. Two ml from the overnight culture was used to inoculate 50 ml liquid media and incubated at 30 °C for 5 h, shaking at 20 g.

2.3.3 *E. coli* growth conditions

A single colony from *E. coli* streaked on LB plates were used to inoculate LB liquid media, and incubated at 37 °C overnight, shaking at 20 g.

2.3.4 Glycerol stocks

Fungal spores, yeast and bacteria were stored in 15 % glycerol at -80 °C for long term storage. Liquid suspensions were mixed with equal volume of 30 % glycerol. The frozen glycerol stocks served to inoculate fresh cultures.

2.4 Antibiotics

The antibiotics were dissolved in the appropriate solvent, filter sterilised and stored at -20 °C in aliquots. Stocks were diluted as appropriate as shown in table 2-1

Antibiotic	Stock conc.	Working conc.	Supplier
Ampicillin	50 mg ml ⁻¹ (water)	50 µg ml ⁻¹	Melford
Nourseothricin	50 mg ml ⁻¹ (water)	50 - 100 µg ml ⁻¹	Werner –Bioagents
Basta	50 mg ml ⁻¹ (water)	50 – 100 µg ml ⁻¹	Aventis Crop Science
Hygromycin	50 mg ml ⁻¹ (water)	50 – 100 µg ml ⁻¹	Melford
Cycloheximide	50 mg ml ⁻¹ (DMSO)	50 – 200 µg ml ⁻¹	Fisher Scientific

Table 2.1 Antibiotics used in experiments. Table shows list of antibiotics with stock concentration, working concentrations and supplier.

2.5 DNA manipulation, modification and cloning

2.5.1 DNA digestion with restriction endonucleases

Restriction enzymes were supplied and used as directed by manufacturer (New England Biolabs, UK). Typically, 1 µg DNA was digested with 10 units of restriction enzyme and 2 µl of 10x restriction enzymes buffer in a 20 µl total volume at 37 °C for 1-3 h.

2.5.2 Amplification of DNA by polymerase chain reaction (PCR)

The amplification of DNA sequences by PCR typically used a PCR mix that would include DNA templates (100 ng) together with forward and reverse primers (1 µl each from 0.25 mM stock), and 20 µl of DreamTaq™ green PCR master mix (DreamTaq™ DNA polymerase, 2x DreamTaq™ green buffer, dNTPs and 4 mM MgCl₂). For PCR reactions requiring greater accuracy, a Phusion® high fidelity polymerase (Finnzymes, Finland) were used which have a novel *Pyrococcus*-like process enhancing domain. A typical PCR mix included DNA templates (100 ng), together with forward and reverse primers (1 µl each from 0.25 mM stock), and the Phusion® high fidelity polymerase mix (Phusion® high fidelity DNA polymerase, 5 X Phusion® HF buffer, DMSO and 5 mM MgCl₂). Amplified PCR products were visualised using gel electrophoresis on ethidium bromide stained agarose gel. Typical thermocycler (PTC-100 Bio-Rad Laboratories Inc, UK) settings were;

Steps	DreamTaq™ green -standard	Phusion® – high fidelity
1	94 °C for 3 min	98 °C for 3 min
2	94 °C for 30 sec	98 °C for 30 sec
3	55 °C for 30 sec	55 °C for 10 sec
4	72 °C for 45 sec	72 °C for 30 sec
	Steps 2 - 4 repeated 34 times	Steps 2 - 4 repeated 25 times
5	72 °C for 10 min	72 °C for 10 min

2.5.3 Colony PCR

In order to directly amplify from *E. coli*, colonies were picked by lightly touching the edge of each colony with a pipette tip and submerged in a mixture that would include forward and reverse primers (1 µl each from 0.25 mM stock) and 2 x DreamTaq™ green PCR master mix. Colony PCR was used to determine whether *E. coli* colonies contained plasmids of interest. PCR settings were the same with the only exception of having the initial step set at 94 °C for 10 min to allow DNA to be released from the *E. coli*.

2.5.4 Reverse transcription (RT)

RNA was reverse transcribed to cDNA. First-strand synthesis was carried out by heating the mixture of 2 µl RNA (*ca.* 4 µg) with 1 µl oligo dT primers from 0.25 mM stock to 70 °C for 2 min and chilled on ice for 5 min. The denatured RNA and primer were added to a mix which contained 40 mM RNase inhibitor, dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), 1 µl AMV Reverse transcription polymerase (Promega), 5 µl AMV buffer (250

mM Tris-HCl, 250 mM KCl, 50 mM MgCl₂, 2.5 mM spermidine and 50 mM DTT) and 9.5 µl deionised H₂O, and incubated at 55 °C for 1 h. One µl of the resulting cDNA was used for PCR analysis.

2.5.5 Quantitative PCR (qPCR)

Quantitative real-time PCR (qPCR) is a method that uses fluorescent dyes to detect the amount of PCR product after each PCR cycle (Higuchi *et al.*, 1992). This method uses SYBR green I which shows low fluorescence when unbound in solution, but fluoresce brightly when associated with dsDNA in suitable light conditions. The fluorescence signal is monitored during the reaction and its intensity correlates to the amount of product formed (Kubista *et al.*, 2006).

For qPCR amplifications, a Maxima® SYBR green qPCR master mix (Fermentas, UK) was used. A typical qPCR reaction mix would include forward and reverse primers (0.5 µl each from 0.25 mM stock), cDNA template (1 µl), Maxima® SYBR green qPCR master mix (20 µl) and H₂O (18 µl) was used. Throughout the PCR reaction, fluorescence from the DNA-SYBR green complex was monitored by the Stratagene MxPro detection system. Typical qPCR thermocycler temperatures were;

Steps	qPCR SYBR Green
1	95 °C for 10 min
2	95 °C for 30 sec
3	55 °C for 1 min
4	72 °C for 1 min
	Steps 2 - 4 - repeated 40 times
5	95 °C for 1 min
6	55 °C for 30 sec
7	95 °C for 30 sec

For data analysis, relative quantitation was used, which determines the ratio of a target molecule in a sample, and in a reference sample. Results from the quantitation are reported as relative differences between the samples. This methodology is widely used when analysing variation in expression levels between different samples (Principles of qPCR -Finnzymes, Helsinki, 2009).

2.5.6 Plasmid DNA preparations

Qiaprep plasmid mini kits and midi kits (QIAGEN Ltd, UK) having spin columns that contained unique DNA binding silica gel membranes were used for small and medium scale plasmid DNA extractions. The mini kit columns collected up to 20 µg DNA while the midi kit column yielded 100 µg DNA. Following manufacturers recommended methodology, plasmid DNA extracted from *E. coli* was resuspended in TE at 1µg µl⁻¹.

2.5.7 Plasmid DNA extraction from *Saccharomyces cerevisiae*

Yeast plasmid DNA was extracted as described by Singh and Weil (2002) with slight amendments. Yeast cells grown in 10 ml YSDOM for plasmid extraction were collected at 260 g for 5 min and washed with 0.5 ml deionised H₂O. 200 µl yeast lysis buffer (Triton X-100 4 ml, 10 % SDS 20 ml, 5 M NaCl 4 ml, 0.5 M EDTA 400 µl, 1M Tris 2 ml in 200 ml deionised water), 200 µl phenol: chloroform: isoamylalcohol (25:24:1) and 0.3 g of 425-600 µm acid washed glass beads were added and vortexed for 10 min to disrupt the cells followed with the addition of 200 µl TE (pH 8.0).

The solution was centrifuged at 20 000 g for 5 min and the aqueous phase transferred to a clean tube and DNA precipitated by addition of one tenth volume 3 M sodium acetate (pH 5.5) and 1 ml of 96 % ethanol, and incubated at -20 °C for 15 min. The pellet formed by centrifugation at 20,000 g for 20 min was resuspended in 400 µl TE and 4 µl RNase A (10 mg ml⁻¹) and incubated at 37 °C for 5 min followed by addition of 10 µl of 4 M ammonium acetate and 1 ml of 96 % ethanol, and further centrifugation at 20,000 g for 2 min. The precipitate was washed with 500 µl 70 % ethanol, air dried and resuspended in 20 µl distilled water.

2.5.8 Sequencing and analysis of DNA

Small scale plasmid DNA preparations were used for sequencing reactions. Prepared DNA (100 ng) with selected primers were sent to AGOWA – Germany for sequencing. Analysis of sequence chromatograms were assisted by Sequencher 4.6 (Gene Codes Corp., USA) and Clone Manager 7 (Scientific

education central) software. Sequences were also aligned and subjected to functional BLAST (Altschul *et al.*, 1990) analysis on the NCBI online software, <http://www.ncbi.nih.gov>.

2.5.9 Agarose gel electrophoresis

Gels were cast and run using Bio-Rad Laboratories (UK) electrophoresis equipment. 1 % and 0.7 % (w/v) agarose (Bioline, UK) gels were used to separate DNA fragments. 1x TAE (40 mM Tris acetate, 1 mM EDTA) was utilised in gel preparation and as a running buffer. Agarose and 1x TAE mixtures were gently heated in a microwave oven. Ethidium bromide was added to the melted gel at a final concentration of 0.5 $\mu\text{g ml}^{-1}$ before being poured into appropriate gel mould with an inserted comb. One-tenth volume orange G (40 % w/v sucrose, 0.15 % w/v orange G) loading dye was added to samples before being loaded and electrophoresed at 100 V to separate DNA fragments. Size-marker DNA (Hyperladder I, Bioline, UK) containing fragments of known molecular weight and quantity was loaded adjacent to samples allowing band size and concentration to be determined (Fig 2-1). Bands were visualised on a UV transilluminator, with the images manipulated and photographed using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc., UK)

HyperLadder™ I

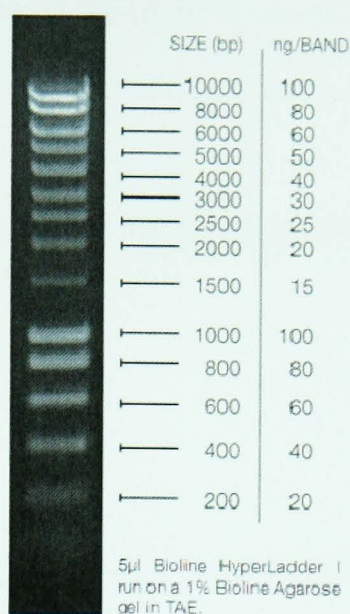


Figure 2.1 Hyperladder I (Bioline, UK. www.bioline.com). Concentrations and sizes on the right correspond to 5µl of loaded marker, eletrophoresed on a 1% agarose gel shown on the left.

2.5.10 DNA purification from gels

DNA fragments selected under UV illumination were excised from agarose gels using a razor blade and purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corp., UK) according to manufacturers' instructions.

The extracted DNA fragments were solubilised for 10 min using Solution A (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate pH 5) at 55 °C to release DNA from the gel. The mixture was then centrifuged at 10,000 g for 1 min in a column that contains a silica matrix which binds to the excised DNA fragment. The bound DNA was washed with 500 µl Solution B (80 % v/v ethanol, 10 mM potassium acetate pH 5.0, 16.7 µM EDTA pH 8), and residual

wash buffer was removed with an additional centrifugation at 10,000 g for 2 min. The DNA was eluted with 25 μ l deionised H₂O.

2.6 Nucleic acid extraction from fungal mycelium

2.6.1 Genomic DNA extraction

Fungal genomic DNA was extracted as described by Schouten *et al.* (2002) with slight amendments. Lyophilized cultures (500 mg) were homogenised with mortar and pestle in liquid nitrogen and transferred to 1.5 ml tubes. Three ml TES (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 2 % w/v SDS) and 100 μ l of Proteinase K (20 μ g μ l⁻¹) were added and incubated at 60 °C for 1 h, and 1 ml 5 M NaCl and 250 μ l 10 % w/v N-cetyl-N,N,N-trimethylammonium bromide (CTAB) was added and incubated for another 1 h at 65 °C. Proteins were extracted with 5 ml phenol: chloroform: isoamyl alcohol (25:24:1) and the aqueous phase transferred to a new tube. To precipitate excess polysaccharides, 1.5 ml of 7.5 M NH₄Ac was added, vortexed and the chilled on ice for 30 min. The solution was moved to a new tube and the DNA pellet formed by adding 0.7 vol isopropanol, and washed with 70 % (v/v) ethanol. The pellet formed was dissolved in 500 μ l TE (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) and RNase A (2.5 U), incubated for 30 min at 37 °C and precipitated with one tenth vol 3 M NH₄Ac and 2 vol ethanol (100 %). The air dried pellet was dissolved in 200 μ l TE. The purified DNA was visualised using a transilluminator.

2.6.2 RNA extraction

Fungal RNA was extracted as described in the Qiagen RNeasy handbook (2006). Freeze dried mycelia (100 mg) was ground in liquid nitrogen, resuspended in buffer RLC and loaded into a shredder column to remove cell debris. The supernatant was mixed with an equal volume of ethanol and centrifuged onto an RNeasy spin column. The membrane was then subsequently washed with buffer RPE and the concentrated RNA eluted with H₂O.

2.7 Transformations

2.7.1 Fungal transformation

Botrytis transformations were performed following methodology and modifications from Hamada *et al.* (1994), van Kan *et al.* (1997) and Choquer *et al.* (2008). Conidia were harvested from 7 day old *Botrytis* plates. The conidia were used to inoculate 50 ml of 1 % (w/v) ME, and incubated at 25 °C shaking at 30 g for 18 h. The germinating conidia were pelleted and washed twice with KC solution (0.6 M KCl, 50 mM CaCl₂) and pelleted. The conidial pellets were resuspended 25 ml KC solution containing the lysing enzyme 0.1 µg l⁻¹ *Trichoderma harazium* (Sigma) and incubated at 25 °C, shaking at 10 g for 2.5 h to form protoplasts and filtered through 40 µm mesh filters to remove any unprotoplasted hyphae. Protoplasts were carefully pelleted by centrifugation at 1,000 g for 10 min at 4 °C, washed with cold KC solution

and resuspended in 1 ml cold KC solution. The protoplasts were counted using a haemocytometer and light microscope.

The mixture containing 1 $\mu\text{g } \mu\text{l}^{-1}$ DNA (100 μl) and 5 mM spermidine (5 μl) was chilled on ice for 5 min. Protoplasts 1×10^6 (100 μl) were added to the mixture and chilled on ice for 5 min. 100 μl PEG solution (25 % w/v PEG 3350, 10 mM Tris-HCl pH 7, 50mM CaCl_2) was added and incubated at room temperature for 30 min, more PEG added (500 μl) and incubated for a further 10 min, centrifuged at 1,000 g at 25 °C and resuspended in 1 ml cold KC solution.

The protoplasts were added to 100 ml molten SH agar (48 °C), poured onto 5 Petri dishes and incubated at 25 °C for 24 h. An equal amount of SH agar overlay containing an antibiotic (e.g. 50 $\mu\text{g ml}^{-1}$ hygromycin B) was added to the developing cultures for selection. Emerging colonies were purified to reduce likelihood of heterokaryons by subculturing 3 times on MEA plates containing the antibiotic (e.g. 100 $\mu\text{g ml}^{-1}$ hygromycin B) with successful monospore colonies being transferred to MEA plates for further analysis.

2.7.2 Yeast transformations

Yeast transformation was as described by Raymond *et al.* (1999). About 3 ml YPD liquid media was inoculated with a single colony from yeast streaked on YPD plates and incubated at 25 °C shaking at 135 rpm until concentrations reached 5×10^6 cells ml^{-1} (14 h), diluted with 50 ml liquid YPD and incubated until cell concentrations reached $5 - 7 \times 10^6$ cell ml^{-1} (5 h). This allowed at least two cell divisions. The cells were collected and washed in 10 ml deionised H_2O and resuspended in 300 μl deionised H_2O . In new tubes, 50

μl denatured (5 min at 95 °C) salmon sperm DNA ($2\ \mu\text{g}\ \mu\text{l}^{-1}$), $2\ \mu\text{l}$ linearised pYES2-19 vector ($1\ \mu\text{g}\ \mu\text{l}^{-1}$), $5\ \mu\text{l}$ antibiotic cassette ($2\ \mu\text{g}\ \mu\text{l}^{-1}$), $5\ \mu\text{l}$ left arm fragment ($2\ \mu\text{g}\ \mu\text{l}^{-1}$), $5\ \mu\text{l}$ right arm fragment ($2\ \mu\text{g}\ \mu\text{l}^{-1}$), $50\ \mu\text{l}$ *S. cerevisiae* ($1 \times 10^6\ \text{cell ml}^{-1}$) and $272\ \mu\text{l}$ Lithium acetate/ PEG premix ($240\ \mu\text{l}$ 50% PEG 4000 and $32\ \mu\text{l}$ 1 M Lithium acetate) was added respectively. The mixes were incubated at 30 °C for 30 min and heat shocked for 15 min at 45 °C. Cells were pelleted by centrifugation at 460 g for 2 min, resuspended in $200\ \mu\text{l}$ of deionised H_2O , spread over YSDOM plates at $20\ \mu\text{l}$ and $170\ \mu\text{l}$ for each transformation and incubated at 25 °C for 2 days. Selected emerging colonies were checked with colony PCR and grown in 10 ml liquid YSDOM for yeast plasmid extraction. Plasmids were replicated in *E. coli*.

2.7.3 Transformation of chemically competent bacterial cells

DH5 α^{tm} chemically competent *E. coli* cells were thawed on ice before adding 1-2 μl of plasmid. The cells and plasmid were chilled on ice for 20 min, heat shocked at 42 °C for 45 sec and chilled on ice for 2 min. To the same tube, $500\ \mu\text{l}$ SOC media was added, incubated at 37 °C, shaking at 135 rpm for 1 h. Aliquots of the cells were spread on LBA with appropriate antibiotics and X-GAL ($80\ \mu\text{g ml}^{-1}$) and IPTG (0.5 mM) for blue/white screening. Plates were incubated at 37 °C overnight. Typically, 10 emerging colonies were subjected to colony PCR.

2.8 Transfections

2.8.1 Mycovirus transfection

The introduction of mycoviruses into fungal protoplasts followed the same methodology as fungal transformation with the exceptions being 30 min waiting period after the first addition of PEG with partially purified mycoviruses or mycovirus infected protoplasts to replace DNA.

2.8.2 Partial purification of *Botrytis* RNA mycoviruses

Ten grams of frozen mycelium submerged in liquid nitrogen were grounded in a mortar and pestle, resuspended in 20ml of 0.1 M Na₂PO₄ (pH 7) and 0.5 vol chloroform, chilled on ice shaking at 20 g for 20 min. The aqueous phase was moved to a new tube with 2 % NaCl and 8 % PEG 8000 and chilled on ice for 1 h. The solution was centrifuged at 4,000 g for 10 min at 4 °C to remove cell debris, and the viruses pelleted by ultracentrifugation at 70, 000 g for 90 min at 4 °C. The pellet was resuspended in 500 µl 20 mM Na₂PO₄ (pH 7).

To examine virus morphology, a negative staining technique which uses heavy metal salts to enhance between the mycovirus image and background was used following methodology according to Palmer *et al.*, (1998) with modifications. A Formvar-coated EM grid was submerged in 10 µl of mycoviral suspension for 3 min. The grid was removed and submerged in 2 % potassium phosphotungstate (pH 4) for 1 min. The grid was then examined in a transmission electron microscope at 80 kV.

2.9 Nucleic Acid Blotting and Hybridization

2.9.1 Labelling of DNA probes

DNA probes used to hybridise to immobilised DNA bound to nylon membranes were PCR amplified with appropriate primers, separated by agarose gel electrophoresis and purified. Deionised H₂O was added to 25 ng of template to obtain a final volume of 47 µl. DNA were denatured at 100 °C for 2 min followed by 2 min on ice. Following manufacturers specifications (GE Healthcare UK Ltd., UK) the DNA fragments were radiolabelled with 2 µl Redivue™ deoxycytidine 5'-[α-³²P]- triphosphate, triethylammonium salt using Ready-To-Go™ DNA labelling beads (-dCTP) and purified using spin-column chromatography (ProbeQuant™ G-50 Micro Columns).

2.9.2 Blotting of nucleic acids

Southern blotting was performed according to Sambrook *et al.* (1989). Ten micrograms of DNA was digested with the desired restriction enzyme and separated on a 0.8 % w/v agarose gel overnight at 50 V. The gel was depurinated with 0.25 M HCl for 15 min, denatured with 0.5 M NaOH for 30 min and rinsed in H₂O. For northern blotting, RNA was separated on 0.8 % w/v agarose gel and run for 30 min at 100 V and rinsed twice with H₂O. DNA and RNA were transferred to a Hybond-N+ nylon membrane overnight using conventional liquid capillary transfer methodologies. The membranes were rinsed with 2 x SSC (3 M NaCl and 0.03 M sodium citrate, pH 7)

exposed to UV-crosslinker (CL-1000 Crosslinker, UVP Inc., USA) for 2 min to immobilise the nucleic acid on the membrane. The membrane was stored at 4 °C.

2.9.3 Hybridization of filters

Filters were hybridised with slight modifications according to Sambrook *et al.* (1989). Filters were prehybridised for at least 1 h in 100 ml Church buffer (0.25 M NaHPO₄ pH 7.2, 7 % w/v SDS, 1 mM EDTA) at 65 °C, then replaced with 50 ml Church buffer and the radiolabeled DNA probe that had been denatured at 100 °C for 3 min and chilled on ice for 2 min. Hybridization was for 16 h at 65 °C.

2.9.4 Washing filters and developing autoradiographs

The washing buffers were used to wash the hybridised filters until Geiger counter rates were approximately 50 - 100 counts per second. The first wash was with Solution A (2 x SSC, 0.1 % w/v SDS) for 1 min followed by the second wash with Solution B (0.5 x SSC, 0.1 % w/v SDS) for 5 – 15 min before being transferred and wrapped in cling film. The filters were placed in a radiography cassette and exposed to autoradiography films (Hyperfilm MP, GE Healthcare UK Ltd, UK) at -80 °C for an appropriate length of time then thawed at room temperature. The films were soaked in developer for at least 5 min, rinsed in H₂O, soaked in fixer for 5 min and rinsed in H₂O before being dried.

2.10 Online resources and databases

2.10.1 Phylogenetics

The ClustalW2 (Thompson *et al.*, 1994) analysis program available online (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) was used for multiple sequence alignment and to produce phylogenetic trees. Protein sequences were uploaded using a standard multiple alignments format (.aln file) and analysed using default options. For the software to construct the phylogenetic trees, pairwise genetic distances between the submitted sequences were estimated and used in a neighbour joining algorithm (Saitou and Nei, 1987). A pairwise score is calculated for every pair of sequences that gives the best alignment divided by the number of residues compared. The alignment score was calculated from separate pairwise alignments as designed by Wilbur and Lipman (1983).

2.10.2 Genome browser

Genome browsers for *Botrytis* give graphical interface for display of information from genomic databases and the ability to browse the entire genome. Annotated data is available that includes information regarding gene prediction and structure, regulation, variation, expression, proteins and comparative analysis. The two sequenced *B. cinerea* strains and their genomic browsers are;

- *B. cinerea* B05-10 is available at http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html. A project completed by Syngenta (UK) and submitted to Broad Institute of Massachusetts Institute of Technology (USA) and Harvard University (USA).
- *B. cinerea* T4 is available at (<http://www.genoscope.cns.fr/spip/Botrytis-cinerea-whole-genome.html>). A project by Bayer CropScience (Germany) and United Research for Genomic Information (France).

2.10.3 BLAST search

BLAST, Basic Local alignment Search Tool (Altschul *et al.*, 1990) searches local similarity between sequences from databases that have been made available online. The program compares primary protein and nucleotide databases and calculates the statistical significance of the matches. BLAST is available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

2.11 Plant, fruit and vegetable bioassays

2.11.1 Preparation for bioassays

Selected plant seeds (Fothergills Ltd, UK) were sown in sterilised Levingtons F1 compost either by broadcasting or individually and watered daily in the greenhouse. The plant leaves or whole plants were ready for bioassays between 7 – 14 days after germination depending on the plant. The fresh

fruits and vegetables purchased from a local supermarket (Sainsbury) were surface sterilised with 1 % domestos (e.g. Domestos, which had the active ingredient sodium hypochlorite) for 5 min and rinsed in deionised H₂O before being used in bioassays.

2.11.2 Lesion diameter bioassays

Assays to determine lesion diameter on leaves, fruits and vegetables were carried out in trays (14'' x 8'' x 3''). One sheet of paper towel layered the bottom of the tray. Twenty ml of deionised H₂O was added to create a moist environment. The leaf, fruit or vegetables being assayed were positioned in the centre of shallow plates (e.g. one side of Petri dishes) which were placed on the moist tissue in the tray, and the tray covered with cling film and incubated on the lab bench at room temperature. Whole plant bioassays were examined in the containment area of the greenhouse with controlled conditions of 20 °C and >70 % humidity.

To allow *B. cinerea* infection for the detached leaves, punctures were made on both sides of the midrib. For the fruits and vegetables, they were halved, and the opened side faced down, with the top side punctured.

The leaves, fruits and vegetables were punctured using a blunt needle and 10 µl of *B. cinerea* conidia suspension (500 conidia µl⁻¹) dropped onto the punctured area. Data of lesion diameter from assays were collected after 5 - 7 days inoculation by measuring radial distance (mm) using a ruler.

2.12 Statistical analysis

For statistical analysis of the data, the one sample *T*- test was used. The *T*-test is a parametric statistic for independent samples, which compares the difference between two means in relation to the variation in the data, expressed as the standard deviation of the difference between the means with a significant p-value of $p = 0.05$. The statistical analysis results were computed by the SPSS 8.0 statistical analysis software.

2.13 Microscopy

2.13.1 Light Microscopy

The Leica DMLB light microscope (Leica Microsystems GmbH, Germany) was used to visualize fungal protoplasts and growth.

2.13.2 Transmission electron microscopy (TEM)

The JEM 1200 EX transmission electron microscope (JEOL, Japan electron optics limited) was used for magnifications at x150 k (80 kV). Preparations of samples for TEM are detailed in 2.8.2.

3 Dicer Gene Disruption

3.1 Introduction

If mycoviruses are to be considered as part of a control system for *B. cinerea* as mentioned in Chapter 1, more understanding is needed about the RNA silencing mechanism in *B. cinerea*. The RNA silencing mechanism acts as a cellular defence system to protect against exogenous RNA including RNA mycoviruses. The DICER proteins which are the focus of this chapter have been reported to play an important role in RNA silencing in other *Ascomycete* fungi (Catalanotto *et al.*, 2004).

To fully understand a gene function, there are well established genetic methodologies available, which include gene disruption and gene knockdown. Gene disruption, also known as gene knockout, is a genetic technique that allows a mutant to be generated with the targeted gene(s) disrupted. The mutants can be studied morphologically, physiologically and also subjected to further analytical experimentations such as further mutations.

To disrupt a targeted gene, a gene disruption cassette is constructed having a selective gene marker flanked by regions from before and after the targeted gene. The cassette is then transformed into the organism to replace the targeted gene. This is assisted by the cellular processes in the nucleus that recognize the DNA fragment and incorporate it into the genome by homologous recombination.

The discovery of RNA silencing and the use of gene knockdown have provided a means to study gene(s) without gene disruption (Baulcombe,

2004). This method has been used widely and has demonstrated enormous potential. However, since this study is focusing on the Dicer gene, which is presumed to be involved in RNA silencing, the option of gene knockdown was not applicable.

In *Drosophila*, *Dicer-1* is involved in miRNA production while *dicer-2* is involved in RNA production of siRNA (Lee *et al.*, 2004). Dicer genes have been studied in several *Ascomycete* fungi. The results showed that *N. crassa* has two redundant Dicer- like genes (*Ncdcl-1* and *Ncdcl-2*) for RNA silencing (Catalanotto *et al.*, 2004). For *Cryphonectria parasitica*, *Aspergillus nidulans* and *Magnaporthe oryzae*, although two genes are present, only one Dicer gene, *MDL-2*, *dcl-B* and *dcl-2* respectively, was found to be involved in RNA silencing, and it should be noted that *A. nidulans dcl-A* is atypically short (Segers *et al.*, 2007; Hammond *et al.*, 2008; Kadotani *et al.*, 2004).

3.2 Aims

The experiments presented in this chapter aimed to investigate what Dicer-like genes were present in the genome of *B. cinerea* and to determine what roles if any they might play in gene silencing. Therefore the aims were to;

- Identify and characterize possible Dicer genes using bioinformatic analysis
- Create appropriate Dicer disruption constructs for *B. cinerea* Dicer gene(s)
- Transform the Dicer disruption constructs into *B. cinerea*, and carry out molecular analysis to identify possible Dicer mutants.
- Analyse growth rates, sporulation rates and virulence levels for the transformants.

3.3 Results

3.3.1 Bioinformatic analysis of Dicer 1 and 2

The first objective of this research was to identify the Dicer -like genes within the genome of *B. cinerea*. Several different Dicer genes including those from fungi, a plant, an insect and a nematode were used to analyse the two *B. cinerea* genome sequences, using BLASTp and tBLASTn. This identified two possible Dicer genes in *B. cinerea* (NCBI Accession number: *Dcr1*; BC1G_10104, *Dcr2*; BC1G_10438). The *Dcr1* gene coding region has 5529 bp which translates into 1842 amino acids, while *Dcr2* has 4197 bp which translates into 1398 amino acids.

The DICER 1 and 2 protein sequences for *B. cinerea* and selected *Ascomycete* fungi (*Sclerotinaceae sclerotiorum*, *Neurospora crassa*, *Magnaporthe oryzae*, and *Aspergillus nidulans*) were obtained from NCBI and their database websites and aligned using ClustalX (Larkin *et al.*, 2007) and GeneDoc (Nicholas *et al.*, 1997). Initial alignment showed pattern irregularities among the DICER protein sequences. Careful observation and database searching identified that the DICER protein sequences of some of the fungal databases were incorrectly identified. The introns were either not correctly annotated or miss-called giving incorrect protein predictions. Therefore, improvements to the predicted protein sequences were made to DICER 1 for *S. sclerotiorum* and DICER 2 for *B. cinerea*, *N. crassa* and *M. oryzae*. The revised sequences aligned for the DICER 1 and 2 protein sequences showed the expected motifs which were characteristic for the DICER proteins (Fig 3.1). The order for the motifs from left to right was DeadBox, Helicase, RNase-a, duf 83 and RNase -b. The roles for the motifs in DICER are explained in Chapter 1.

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A_n_Dcr1 : -----
B_c_Dcr1 : MIRDAAAASLYHWRKGVTPSAEEDLLSFDDIVTAVPTTSSSVAPYTSRDKTPSASGNGDAIADSSGYLKQAVSSHSAVRSSSNNGQDAKSSPSISPDSKLEIFGPPLREPSPKPFNKS--ADTFDKSSLAKTADMDSFVSHHADVVISLGSANQIPSGNISTQDLS--PSLENHSPNVT
S_s_Dcr1 : -----METNAYHOTASKQSSSDEEDLLSFHDAVTVIQPVSSSVNVDTRFRGNISSFAHSGNNAVVGNSGINKVQSSYSTSSSFLNKGQDGTGSLSTLSTCRPEIIFGSPENPISLHKISPPAGTKGLSLTRIASMSPQAPHVADAAMTNSLCPDLYRDIPIQPGSSFPVSSAVVSAT
N_c_Dcr1 : -----MAVATRLPPTPEATSGIIGGEDLLDLSQEDVSDNDGRNASDSEDGKVRWTVNPEP-KPKKISAKKLADTAFAFWIEHQAELAR-----DQRKAAEAARVAG--VVLPAIGFDSERHITSPEYQVLEPERAKQQTAVLDGTGSKTILTAVALRWVITGELEDREKGLPR
M_o_Dcr1 : -----MEVHDGLKSPDKAAKSRYD-----DDR---IIGDSEDAVRLVANPDPSPKPKISERKRADTAFAFWNDNKIRLSLPSATKSRRSGIRELFSDEITETRPRTREPRERIEGPREYQIELPERAKQQTAVLDGTGSKTILTAVALRWVITGELEDREKGLPR
A_n_Dcr2 : -----MQPALQVLHDSLLAAQHLSDHPQPPRQPPNAQPHHLQPTATTSPRQNNIDPAISGGAMIPPSQPPAQPEPTVEDTPKTYGKRPLSTSKRAAQNRATRAFRQRKESYIRKLEEQVKEYVMSQYKALQENYQIRBYVNLQSRLLDRTEISVPQPAPRPGQAGASAPPQGSQSQSVS
B_c_Dcr2 : -----KEY-----SEPDTDPDTGSLIDGRDIEGLIALTSGERINETVELLCSDSGLIVENEDDNGAGKGEIVITPTTYOLEMLEESLKRNVIVAMDTGSGKTHAVVIRLAEELERMKPKG-----
S_s_Dcr2 : -----MDNCTRNQKIPVAKNALFEPHPOFISQTDIIPSKIASNPAYPDIGSGSLVDDQHEVEGYLDGLTSGKISNEIVSLCSSTHEAIGNED-AISEEGKGNVITPTTYOLEMLEESLKGNIIVAMDTGSGKTHAVVIRLAEELERMKPKG-----
N_c_Dcr2 : -----MDQDPRKDNPEMDVDRDDSSDPDDNESFKSADEPLDKELYDALEKPSPEEPSEELNDQVSTPA-----ALTAAYQLEFASLIKQNIIFSR-----VMVADGHWQWQDSG-----
M_o_Dcr2 : -----SSQDESASSSDTGEGGHVFGRLNLSADYGYKTGSSASTHSTTDDAIS-----EPQPSGNGPR-----SHIAAYQLEFASLIKQNIIVSMDTSGSKTQAVVIRLAEELERTPEP-----

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DeadBox

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A_n_Dcr1 : -----MEQINLVFDEAHHTKKDFVCARIIRDSYFETAQSKR--FFVIGMTASPVDTG-----DVAQAMMLELYLDSKUATASQLSLVHQVVRFP--SEAWITREEQSFGDTYRL
B_c_Dcr1 : PINKVPT--FELSTRITDDFAELRRPIH--IFDVFLKEATKLPTTEKTSCHANDEATOSTRWLIDQPEKQHITESPRDYQLELEKAKKQNIHPYARIIKDFYRNDTEKDIAFKIFGMTASPVLEARD-----NVKKAABLEGILHSHQICAFEDPSLQYS--IKGAPETIAYDIPGPKNTPIYLQ
S_s_Dcr1 : RITQASMP--FALATRTTDDFAELKREHAQIFDTFLKEVAKPATEKISHADDEAVOSTRWLIDQPEKQHITESPRDYQLELEKAKKQNDHPYARIIKDFYRSDTDEPISIPKIFGMTASPVLEARD-----NIKKAABLEGILHSHQICAFVDPSSLMQYS--IKGAPETIAYDIPGPRFHPPIYLK
N_c_Dcr1 : RIAFFIVDKVALVFOCHSELTKNLDFEYK--LCCEMVEG-VESKAFKEATEQNEVVCATBILSTATHHSWIP--MDQINLVFDEAHHTKKDFYARIIKNFY--IDEQLER-RPRILGLTASPVLEAK--V--DPRRAASLEAMHSHQIATPADPAATQHTIGKATELVNVEVRGRPDSEIVNKO
M_o_Dcr1 : RITEFIVDKVALVROQTDHIRANLDFEYTG--LHCDTVRLLYSKEYEKLQEQEVVVCATBILYRCIHRSYLN--ISQVSLVVFDEAHHAKNHYARIIKDFYLMEECDQK-RPRIFGMTASPIDTKETCI--SYERATHGLESILHSEIATISDRDLIKVIGSRPQ--EVRKSYARLROENTELCNQ
A_n_Dcr2 : ILNRIAEAGLGRKHPNDEAFLSNNFQARPGRGDEATDPSEKTEPEPTHGPMSEWFLSGDLSLADSDNPMASIPBERSLTIIFT-AHNOVNKAPGAKIMTAFYHAYKSSISPLPHILGLSASIPQSPAYHTRAGELGWEDKVELWTEQAWDVLIEGLQIVSTPAVLHDAWGHGFEYNSVCTPQAH
B_c_Dcr2 : -LIWFLAPTVALCACHHEYLQNLIPSVLIKMLICATGVRWTEQONDTVLKQVKVVSQYVLLDAITHGTVR--MGRSLIIFDEAHNOLNAPGAKIMTSFYHE--YKSIFPLPHILGLSASPVMRSS-----PQSLSDIETLEAICCPKIHRAIDRVRVKLPILLSIYITP---ESNIIVTHT
S_s_Dcr2 : -LIWFLAPTVALCTCHNEYQLNHSVILVKLIVSEGVDRWTEQONDTVLKQVKIAMSPPQVLLDAITAHGEVP--MERLSLIIIFDEAHNOLNAPGAKIMTSFYHE--YKSISPLPHILGLSASPVIRSE-----PQSLSKIETLEAVCRTPKHQADIRVRVKLPILLSIIEYNP---EPEYIMTKT
N_c_Dcr2 : -RIWFLTPTVALARQHRVLSQSPSVKAIMCCQIGVDSWSEQAVMLAVLINRVVSTYQIILEDANHSFVR--LDSLSTLIVLEAHNOLNAPGAKIMTAFYHAYKSSISPLPHILGLSASIPKSN--LADIEKLEQVLEAVCRTPITHREELAHNRPEMLAVSYGDSGTDP--TPIDL
M_o_Dcr2 : -RWVFLAPTVALCAQGYFVIRSQKVASIVMTDGNVDSWSDVOITLAVIANRVVCTYMLLEALSHAFVT--VDSLSTLIVLEAHNOLNAPGAKIMTAFYHAYKSSISPLPHILGLSASPVMRSS-----LSSMEDLESSLDSVCRGPILQREELFANHQPTMTVTYKEDCLRSVPQYITN

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A_n_Dcr1 : MEGRUGDIEDLKIIVEREAWQSFELC-RWCSIGVLRYVFSAKMIPKLECK-----SKELSQRPDASEWASSYILGSEE-----EPGQSHKQVLRRTTEHRETPEP---RSLVFTTRFYITIMLELFNALSMPLHREGILIEVILTSS-
B_c_Dcr1 : MLPLDKDNPIFFKPEVFGTEASITLC-SWQVDQITFCLQEEESKKIARTEQAAHKKPVPEPLVLEERKEQIQEAKSIVENHTEFPPHFASRL-----LDDFTIKVHYSNNLSKVVALLSTIKDRQFPTN---DKCIVFVKERYTARLLASLSTPAG--TFELKAAPVIGTTS
S_s_Dcr1 : MLPLEENPIFFQKPEIIGKEASITLC-SWCAQDQITFCLQEEESKKIARTEQAAHKKPVPEPLVLEERKEQIQEAKSIVKDHTEFPPHFASKL-----SDDFAIKVHYSTNLSKVVALLSTIKDRQFPTN---DKCIVFVRERYTARLLASLSTPAG--TFELKVAPIVIGTTS
N_c_Dcr1 : LRKIAGGQDIEFKKPLNHTTSPASKLC-TWCAERYQIFFKQEDIVKLESRTED--LMVAALDEITEKHVKQVREPEHLVNAHTESAAL-----DPTMLSSKVMILVRIIRDQFERGVG--AQCGIIFVRQNTAMILLPILQQPEIKSHIESIAEVLVGGGT
M_o_Dcr1 : LRELVGNHPIFFKOTELSAEFATELC-AWCAIKLELCPEEAVSTLDGRVEGS--RANDPEVCESSHEVSNAERELSLVQOWSESSEF-----EDGSLSTKTHKLEITLAECSQASAGNATQCIIVFVRRTAVLINALCEQASIRTKIDIKGAFLIGAGR
A_n_Dcr2 : RQELLEYTHVPEIRRVLYTPLMENASLAEGSTLQKLEPNDIYCSGQKTFVCKA-VHIFQELGIWAAEYFIRASVEELISHAYVHSKIDLDYDEREYLVNLSKSPVPDIDVHSTDPKDFVSEKFEALISFMSTEDINFSG----LIFVEQRFAVIVMSYLLSTHPSTPDRRTGSGFIGMSNST
B_c_Dcr2 : VASIRKIVCSUNIFELPYVITLARSDEKSQREIAKVIKSFKIYSQTOIKSIDKTSNELIIVLELCPWAADYYISTVVIRYKAMSADKTFIVEDSPAEEKL-YIAKALQVEISPTLSDTGKLSNKVEKLLIGTHAQKPPFSA-----IIFVEERATVSVLPHILSHHPLTKDRFKITGMVGTSLNG
S_s_Dcr2 : VASIGKVICUNISELPYVLAKSSSEKSQREIAKLINNYKINSQREIRLIYNTG-KTILVELGSWAADYYISAVVVKYKTKKAREVFVMDLAATERL-YIAKALKQVGISPVFSLADKTSKVAKLNIILQCEPPFSA-----IIFVEERATVSVLPHILSHHPLTKDRFKITGMVGSSSNS
N_c_Dcr2 : MTRFEAYHRDTSKQPDVILLKAQRIERAREKLROHITKDTIAQKELRGYYNRA-LLVREICPWAADYYITVSHMLAEFERGEFPAQHRY-----IGEALRSIPIPAISKEPIQISEKVTILKVLASHQDFVG--IIVFVKERVMSIVTHISTHPLTKDRYTASMITASVP
M_o_Dcr2 : MNHHLAFENDIAEFPYVILHMGQRNPKSLRELDRIITLQDYTONOKAFQKRS-SSIFRELQGVQVSVYNNVEKVLRLRLRG-----YEGSEVG-----IIVFKERATATISQLIAIIPDINAPYKQCMVGTSTRYM

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Helicase

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A_n_Dcr1 : -----SIRIQWKKV-----
B_c_Dcr1 : ASAG--EMHITERSCTLMHNFNRNGKINCLIATSVAEGLDIEDCNLVVRFDIYNTVIOIICSRGRARHINSRYHYHVESHNEDQIRTIKEVLKHGKILKLFASALPEDRKLGNNFNMDYFLRKEGRHRIYPVENSBAITLYMSLTVLSAFVDSLAPAPESLVEDVYVITVVKQ-----FI
S_s_Dcr1 : TSAG--EMHITERSCTLMHDFRNGKINCLIATSVAEGLDIEDCNLVVRFDIYNTVIOIICSRGRARHINSRYHYHVESHNEDQIRTIKEVLKHGKILKLFASALPEDRKLGNNFNMDYFLRKEGRHRIYPVENSBAITLYMSLTVLSAFVDSLAPAQDSALVDYVITVWKQ-----FI
N_c_Dcr1 : TGSSYVNAKINFCQCNRIIRKFKLGEINCLIFATSVAEGLDIEDCNVIRFDIYNTVIOIICSRGRARHINSRYHYHVESHNEDQIRTIKEVLKHGKILKLFASALPEDRKLGNNFNMDYLLRKEKGRQITVEDTGAKLSYMQSIVCIANFTATLHPPETSLSPEYIYITVPG-----FO
M_o_Dcr1 : GGN---AAFTITRQEQTVSRFRDGEINCLIFATSVAEGLDIEGCVVIRFDIHTGTIIOIICSRGRARHINSRYHYHVESHNEDQIRTIKEVLKHGKILKLFASALPEDRKLGNNFNMDYLLRKEKGRQITVEDTGAKLSYMQSIVCIANFTATLHPPETSLSPEYIYITVPG-----FO
A_n_Dcr2 : NKTIMGLLLSARMCPTLDFRFGKNIIVATDVKEGIDVSACSVVICYNIKGFSEICORRGRARRQNSTYSMTSHEDDGS-----TLDKWKFEKIMDEACLEDRRRTTEPRAGSLTIEDVCTR--ICVRSIGAILTAIYAVQILVFEDTLERQNYVEDKPEESFERNDG-----LLR
B_c_Dcr2 : KRTDQIGELYDVNCKDILSSFRGKILILIAINVLEEGIDVACNLVICSFKANLKSFCORRGRARQDQSKLILDASG-----KATVWHELERKMPPEYGYKEMRLQHYETATAEQSEDDVLRNESTGAQDLDSATPHLYFQSVITTKD-FVDLRDEFVYSEIK-----SEYVR
S_s_Dcr2 : KRTQIGELYDLDKQDILSFRGKILILIAINVLEEGIDVACNLVICSFKANLKSFCORRGRARQDQSKLILDPLEG-----KVIDWHELERKMPMYENEMRLQHYETATAEQSEDDVLRNESTGAQDLDNAVPHLYFQSVITTKD-FVDLRDEFVYSEIK-----TDFVR
N_c_Dcr2 : GARNHMDYTKK--EDMTSLEGFRGPNLIVATSVALEEGIDVICHNLVICSFKANLKSFCORRGRARVSVSTLYLVONASS-----SATDWHLELERKMYEDEMRQNAHEIILDPRIGSYP--VLEJESTGARTIERSHINHFCAKSSSRYLQPEYFVIRQVNPDPASPGRRRTLLQ
M_o_Dcr2 : SRKMNHHEVYIQQGDDLLALEKFFSGATNLIIVATSVALEEGIDVICHNLVICSFKANLKSFCORRGRARRMBSKLHLMDEEDSLV-----SVKDWGALDAAKORYENMRLELDHIAQVEEDEMVDV--PLHSRTIAKVIYVDDAKGILHFRVIRVRSQYVDASLEYLVITTK-----

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A_n_Dcr1 : -----
B_c_Dcr1 : CBAILPEEAPIRGAIG---RPATIKQVAKCSAAEFETCVIIHQKEYINDYILSTFKRS-----AHMMRNALAYDGKQKQAYDMOTAPTWASSKCKQGIFFMIVLSIKSEDNIDRASQPTIGLITRSELPFELPEFVLIHFG--AGRNSPVSCVPIASSITTEKNKIDOWNMETICIFQDVFSKANKSPDS
S_s_Dcr1 : CEVILPEEAPIRGAIG---RPATIKQVAKCSAAEFETCVIIHQKEYINDYILSTFKRS-----AHMMRNALAYDGKQKQAYEMSTAPTWLSKEQGIFFMIVLSIKSEDNIDRASQPTIGLITRSELPFVLPQFLAHFG--GGRNSPVSCVPIASSITTEKNKIDOWNMETICIFQDVFSKANKSPDS
N_c_Dcr1 : CEVVMPEASPIKSAVG---KVHLKSGVAKCAAFELCLAILKACHLDNHLQSVFTKQ-----IPMRNAPLAYSSKKTEYAMRLAPELWSVRGVVIOLEAFVLENEEDTIGRSSRPIILLRSALPEVASFPLEFG--TKRFKVRGVPIPGSVQADDTIVEQLTRFKAQMDVFSKANEATAVN
M_o_Dcr1 : GEVYLEASPLPSAMG---GVIRSKQLAKCAAAVAMCIQLINSNYLDHLKSTLAKV-----IPAMRNAPLAYSSKKRSYNMRTAPVLSVSEVCPITELYAMVLSIAQGAAYYHSRPIILLTRKLPFELRQFFLFGKGSRRSNVRGPIAHFWSPTTTQEGICATTECFRDI FSKDQASGTD
A_n_Dcr2 : PKVILESSVNPAYRRAGKAWAKTERAAKKEAAFYAYKLYEHGLVNDNLLPI TKSR---EFTRKDISLLPAVQKYSECYDFVVDWAHLWSS-----INLYQNRILNRQNEED---TSKFTTPTATPEIAPMKLQWT---SETTYTTEFEAAGAVSLTAENTERRAATSYTQATTSTPLAGNKDY
B_c_Dcr2 : PKVILPGSVSKPIRPHESRGSLSEKSAKDAAFEAYSALYRGGLVNDNLLPLMVHCKVIDELTSKPVDTASLLEVKERLNPIDIRANKEAEHHAGIVRSIMMIFNG-----MKTEICLFDPEATPELKLWY---ADIEFFDFDNDIEIGTSENMTATALNDTNLLS--DRGRVHVQSRR
S_s_Dcr2 : PKVIFELSVETLRPHESRGVALSEKSAKDAAFEAYVALYKGGIVNDNLLPLMVHCKVIDELTSKPVDTASLLEVKERLNPIDFVARANQAEHNPGIVRSIMSLD-----LKTEICLFEPEETPELTHMN---SSNELSVEFANDAEIGTREDMLRALKDITYLLSGATSRREDIFSRR
N_c_Dcr2 : PTVHLEASLAPDRRHESLWTATSEKLAIMDASFQAYKALNAGLVNENLLTKVSD-----FLADIGDD-PGHLWVATQDFAPPEVAYAQE---SSSLYSRRLETVLVIGVE--NPLEFEFILLVEFVYAPLKLWN---ATSAITLITSPEMQEFRKQEGTSAGPDHSYALAMAHAREPIQGRQ
M_o_Dcr2 : -----TAFVGCAGLIINCHLLPTRES-----FVDPEVDGSPGLEVQECWNPFCIVEAMETGLSSQTLHRRRTQUTTODG---LMGENDAILLEVEFEETISILKLYMI---QHSEHPWTISFDDPVITMSGPGSTDPDQTCOLLIDMAHGRETSSEQS

A_n_Dcr1 : -----
B_c_Dcr1 : MEYFLVPIINCLNAIVDWKSN--PMSIIDWETVEYYQDFENKQADKPWEHKPWLGKPIIDZFKDKFITDPDFDSRKLWSVGITKEYRPLDPVPEP-NTAERKGARK---NNSNIMDYSCSLAKAKATAKRTFDEEQVVIDPTYIS--LRRNLDEEDGGELETSSKSRITIEPLKWSPTPTTVGAKAHLLEAT
S_s_Dcr1 : MEYFLVPIINCPNHTGKWDYE--PMSIIDWETVQYVQDFENKQAGKPEHKPWLGKPIIDZFKDKFITDPDFDSRKLWSVGITKDYKPLDPVPEP-NTAERKGARK---NNSNIMDYSCSLAKAKATRRTFDEEQVVEATYIS--LRRNLDEEDTGEVETPKKCVILEPLKWSPTPTTVGAKAHLLEAT
N_c_Dcr1 : LEYFLSEMFG-GHGFLFRLAKSAHLIDRKALAYVSENEK-----VEPYTFL-----EPDEFQDKFVVDPYDAPKFTTHHRRHDMKPTDPVFDGIVAENHRAWGLGTTHTINYSNLSKSKSGFMIQADQVVEAPALIS--TRDFELDTLRDEVDVPOQCSLIEPMLRSPITADYVAHLLECFESI
M_o_Dcr1 : MEYFLASTGIEHGTDLSSLVNBERIIDWATVHRTTTTDR-----VEYNFN---EPDEFQDKVSDPDFDSRKFEMKRVRRDLKQDKVHEGVPAE--SKWR--AVEHTILNYSVSLAKKSR--AGHNSRRQPVVEAPELAPPLRRRNLEDETGENSTGPAN-----IPCPCGNISIPVDTVVVAITTFEAT
A_n_Dcr2 : IALFPGDLPWDELETWIKKQGHepatQVFSSQRPLDRMG-----VVRERSRYGELLIEKRWINRSGDTELECD-----PYPKRRNLELRQTHAK-----KRPATDEILGSPTKKRITLSASHCTIRRHASETVF
B_c_Dcr2 : TVVQFILIQ---DSGSLSSDCFPVDPNGNIKST-----GEIREVGKLESPIIEKWLPE-----NAPEDVEYLAVVKVSR---ADSLHKVQNEKPSSETKQFSSVIPASTCVQDVMEQLSRF
S_s_Dcr2 : TVVQFIANR---APASINLDCVTVTYDEPSRIT-----GEIREFDKLAQPIIEKWLPSKPPVNEVQ-----HTYLNYSNAPENVEYLALIPVTR---TDSLHKVENASPSS--SKQFSCVIPASVCQDAMPFOLVQF
N_c_Dcr2 : YPIRLVSTRKRLVDGIAALEFDPRIYESSQF-----PIVRLVDGRNMPIYFVKILPSKPPVELIS-----KPSTDHADLPENVEYVCK---PIGKAVAAHSPVNHHRELLO---
M_o_Dcr2 : KAIRFLWPIRRTLKLMIGGQPFSSKKAGEHSSD-----YIIRSYGRD--PYIKKSVVEVKPPELVRG-----YIKAKDEEEPACASWVVKRIPNCGFVKNSRIPIELPKYQTSPPRRFDYILPEQLCTFDIVPATIVOL

RNase-a

A_n_Dcr1 : -----
B_c_Dcr1 : IHRVESYLIALEATDILLHDIIRPDALAEVTKLSDNSGDEGEEQTNFCRMENNYERLEFLGDCFLKQGTSSISLYGLNPSDEFRYHVDRCMCHCNKNLENTALKILELYKIRSAENRPAWYPEPELIRGKTAT--APNHHKLGDKKEVADVCEAMIGAPILLSHSHESK--MENAVRAVIEVNSDNH
S_s_Dcr1 : IHRVESYLIALEATDILLHDIIRPDALAEVTKLSDNSEPHGEEQTNFCRMENNYERLEFLGDCFLKQGTSSISLYGLNPSDEFRYHVDRCMCHCNKNLENTALKILELYKIRSAENRPAWYPEPELIRGKTAT--APNHHKLGDKSIADVCEAMIGAPILLSHSHESK--MENAVRAVIEVNSDNH
N_c_Dcr1 : IHRVESNLVALDACKLIGLILRPDLALEFTKLSDNSDDEHDAEKENFCTGMCDNYERLEFLGDSFLKQATTIATYITLTKGFEYHYERMLIICN-NLFNNALEIGLEEYIRSMSENRPQWYPEGLIIKKGKSKD--ARQRHVADKSIADVCEALIGAAYLTGQEKGS--FUMAIKAVTAMVKDKKH
M_o_Dcr1 : IYRLNSLISLEACQNHGDIPTIDIALIPMTKSDNSDDHDEAPINFQSGMCQNYERLEFLGDCFLKQATSIATYITVEGD--FEYHYERMLDICKNLLNWALESNLQHIRSKSENRPWTYPPEGLIKLKGKTE--VDDEHAGDKSIADVCEALIGAAYLQAQASSPNFLAVKAVITVWTHSKTH
A_n_Dcr2 : GREIPVITDRLEAA-LVATSLCETVLRDIQFOLR---HVITAITMPLAQAPIDYQRYEFGDSVLKFTVAASLYNNPNWHEGYLLETLHATVQNAFLRAALDQGLIAYIISNRETPEKKSAPLISEKLYASAS-----TPSMSAKVILADVCEALIGAAYIDEG-----THKAQSCIVRFLFEIEL
B_c_Dcr2 : GMLPISITHHIEVQ-LVVDRLSRITLKDLEISLQ---SLIQTATIHASYSLDSNYQRLFLGDSILKICTSVQVAEHLDAHEGYLSAMKDRIVNSPSSRAAEVGLDEYIMTKETGAKWRMYVDD-LVVTEQ---KTPEMSSKILSDVVEALIGASVWS-----VAAFLKPLNLYN-----
S_s_Dcr2 : GMLPISITHHIEVQ-LIVDRICKITLKDLEISER---GLVQTATIHASYSLDSNYQRLFLGDSILKICTSVQILSEYELAPEGYLSAMKDRIVNSPSSRAAEVGLDEYIITKETGSKWRMYVEDYLKTEQ---KSPQMSKVLSDVVEALIGACMVDCG-----TPKALKSLQFFELN--
N_c_Dcr2 : ---WKLIVNNVRIICRASRF---GDEYIINSAAKKWRPRFVEDYDEMKSPISAETVPNHH-----ERLLSQWKDKIVNNVRIICRASRDFGLDEYIINSAA--KKRREKREVEDYDEMKSPISAETPQSSKMVDVVEESLIGAAAYCGG-----VSKATECVALLPTPKS
M_o_Dcr2 : GMLPISITQAMEPYFAQEDLIRTAIGSLDFKII---DIVMMVLTSSSACRLINRYERVEFLGLAVIKIGAAVTCATNLHTEPEGLISILKDRIVNSRIQATATGLDQFILTQYS-LQQRNMTAPEPPIN-----TPKMSSKITLADVTEALIGAAYTEGG-----VSKALRCISLMIPNER-

duf 83

A_n_Dcr1 : -----
B_c_Dcr1 : NAVVWSYYKLYCKKQYOTATATAAQIDAPQVEMAHPIHFHHPRLIRSAFIHEANLFYIYQTECQQRLEFLGSLILMAOVNFIHNNR--TKCPQALTEHMMAVSNQOFGALCVKLG--EHHLLTIDS-----QVQ---KMTADYSSDINEA-----LITCA
S_s_Dcr1 : KATVWSYYKLYCKKQYOTAVATAAQIDAPQVEMAHPIHFHYPRLIRSAFHEAPESYEQIPSYQRLEFLGDALLIMN---FLHKKYR--SKCPQALL-HMMAVSNQOFGALCVQLGFGHHHMLTIDS-----QVQKMQKMWVEYACDINEA-----LITCA
N_c_Dcr1 : RMISYGQYAWYCKKQYOTESAASACRDVAKKFSERMGYKHHPRLLRAAFCHPTYPSTYERLPSYQRLEFLGALFMMVAVDYLKRFK--AAPQALTEHMMAVSNQOFCCLSEHLG--HNCIATASP-----SIL---KDIAEYVTEIEEA-----LETA
M_o_Dcr1 : TMQAVSYYASYCCEALSTPPSQTOEICSQKKNKMGYRFNPRLLRGAFFHPSYPROYENIPSYQRLEFLGSLILDMVVDYLLKKHP--DKCPQALTEHMMAVSNQOFGCVAVGLG--EHHLLIHYQP-----AIG---GSITEWABLVTKK-----REDA
A_n_Dcr2 : P-----ETKLPRPESYPMKDKHKKPHLIQOENLENHIGYTFRDKTLLMEALHTHPSOPYDTS--IQSYQRLEFLGDVLEMLIVDILRAHHV--ECQQGEMIKIHAIVNGHLLAEFLCMCFKWAMPSLTPSIDTGTEETEIIISP-----PKTILSYSLRYSPSRPLHVEPESGSSNAITR
B_c_Dcr2 : -----YLPRTLAPLRNSTNLTPTSCLRPEVGIHAYTFYTKSLIVBAMTHPSYTSG---TQSLERLEFLGSLILNIIVTAMTSHST--PISHFHLLRSALVNADLAFLCMESIDQVNTNLTEGKNRIHETHS-----RRRVSLVSLPHSSV-RLSIYCKE-----ALSP
S_s_Dcr2 : ---WLPLETRMSLYCLADLHLPIAQRIEFCIHAYTFYTKSLIVBAMTHPSYING---TQSLERLEFLGSLILNIIVTAMSYET--EUSHFQMHLLRSALVNADLAFLCMESIQYQVTLDETEGKHQIHETHT-----RRRFSLVNLPHSS--TESIYCKQ-----ALAP
N_c_Dcr2 : SQFKV-QEIEISRTQLFEFFPKDAILSKQIEPLEKADYTFNKKSLIBAMTHPSCAGLTNESCERLEFLGDAILLVIVVKRMAETGPNEIANDHEHLSSVVIADIMAFAMVWIMQTDINEIDPTNLDALGLLPSSQSRIPTASLVSNKEDWPFWRMRHNSP-QVATITP-----THEP
M_o_Dcr2 : ---W-RSLDESRAVLQVRLN-IPLPSTIGPLEEELGYFNKKSLIIBAMTHINIPGSAGSLERLEFLGSLVLEFVVVAREFSVKDPAPHEHYNHLLTAVVNGEFLGELAMENRTAATQRSVVRTGQGTADELETVET-----PGLPIFGELRNASGDWVRDQCL-----FEER

RNase-b

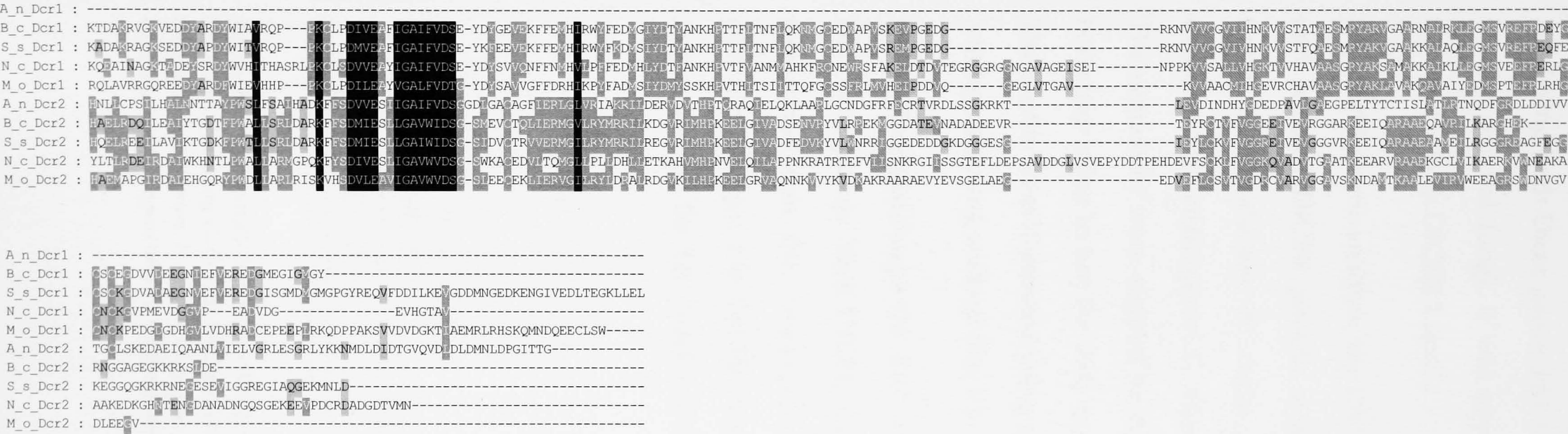


Figure 3.1 Protein sequence alignments for DICER 1 (Dcr1) and 2 (Dcr2) for *B. cinerea* and selected fungi, showing motifs typical for DICER proteins such as the Deadbox, Helicase, duf83 and RNase -a and -b. DICER protein sequences for the fungi were obtained from their databases. A_n: *Aspergillus nidulans*; B_c; *Botrytis cinerea*; S_s: *Sclerotinia sclerotiorum*; N_c: *Neurospora crassa*; M_o: *Magnaporthe oryzae*. Functionally conserved regions (Black), 50 % amino acid identity (Gray). *Aspergillus nidulans* (A_n_Dcr1) has a truncated Dcr1 protein sequence (Hamond *et al.*, 2008).

Analysing both Dicer genes and DICER protein sequences on the databases for the selected fungi, it was apparent that all the Dicer genes had three introns for both DICER 1 and 2.

For phylogenetic analysis, the DICER 1 and 2 protein sequences of *B. cinerea* and selected DICER protein sequences from fully sequenced *Ascomycete* fungi (*S. sclerotiorum*, *N. crassa*, *M. oryzae*, and *A. nidulans*) and other organisms (*D. melanogaster*, *C. elegans* and *A. thaliana*) used as the outgroups. The sequences were aligned by the ClustalW2 software which uses a 95 % bootstrap value to test the data set against a single node (Larkin *et al.*, 2007). The results were illustrated using a phylogenetic tree based on the neighbour joining clustering method (Fig 3.2) (Saitou and Nei, 1987).

It was clear that fungal DICER 1 and 2 proteins form distinct clades for each DICER sequence. Each DICER clade was made up of either DICER 1 or 2 sequences from the *Ascomycete* fungi, with each clade having slightly different lineages. The DICER proteins from the outgroups did not integrate into the fungal DICER clades but formed their own clades.

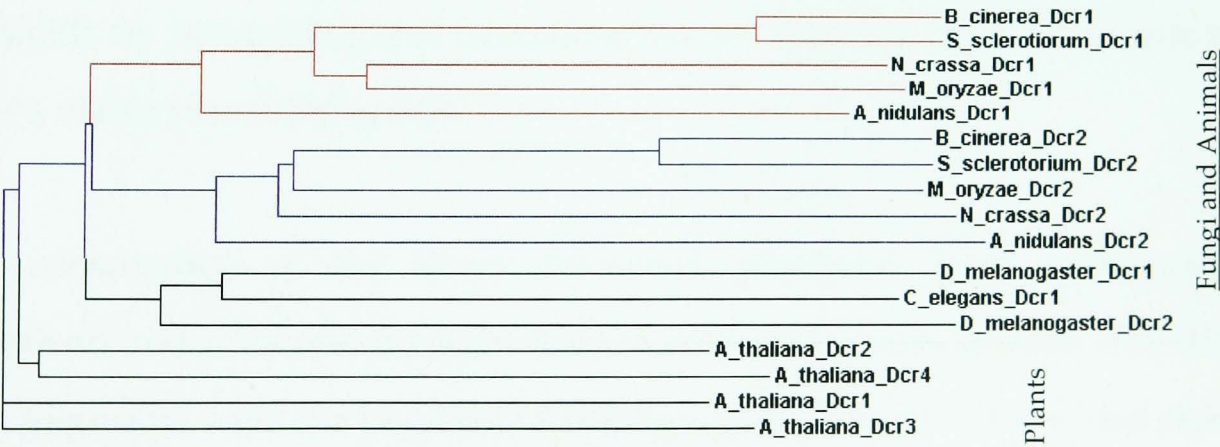


Figure 3.2 Phylogenetic tree (ClustalW2) of the DICER 1 and 2 protein sequences for selected organisms, forming clades for DICER 1 (red) and DICER 2 (blue) for the *Ascomycete* fungi (*B. cinerea*, *S. sclerotiorum*, *N. crassa*, *M. oryzae*, *A. oryzae* and *A. nidulans*), while the outgroups (*D. melanogaster*, *C. elegans* and *A. thaliana*) formed separate clades.

3.3.2 Dicer disruption plasmids pYES2-D1, pYES2-D2, pXMAS-D2

To create the desired Dicer mutants, Dicer disruption plasmids were generated that would enable homologous recombination to replace the endogenous Dicer gene (s) with the selection cassette.

A survey of the available literature shows that disruption in *B. cinerea* could usually be readily achieved by transformation with a selectable marker flanked on each side by targeting regions between 0.5 – 1.5 kb, which typically results in *ca.* 60 % of transformants being the desired disruption mutants (Noda *et al.*, 2007). The initial design for the Dicer disruption plasmids tried to utilise the available restriction enzyme sites, but complications caused by the unavailability of preferred sites in some locations meant that use of restriction enzymes for plasmid construction in this experiment was not practical. Therefore, the alternative was to create the plasmids using in-yeast homologous recombination (Ma *et al.*, 1987). This method utilises the yeasts natural ability to repair DNA breaks by homologous recombination which in turn would be used to create the plasmids by recognising and annealing the overlapping DNA fragments to give a viable plasmid (Fig 3.3).

For construction of the Dicer disruption plasmids, three overlapping fragments and a linearised yeast vector were made for each cassette. The first two fragments were the two Dicer flanking regions named left arm and right arm relating to its locality to the Dicer gene. To design the arms, 12 kb DNA sequences having each Dicer gene was downloaded from the *B. cinerea* database (<http://www.broad.mit.edu>). A 1.5 kb sequence for the left and right arms was selected from upstream and downstream of each Dicer gene

respectively. For *Dcr2*, another set of arms having 2 kb length was also designed.

Forward and reverse primers (Appendix 1) for the left and right arms were hybrid primers containing 25 bp for PCR amplification and 30 bases to provide the necessary homology for yeast-based recombination, and were designed not to create dimerization or hairpins with the PCR products. The arm fragments were PCR amplified from genomic DNA using the primers and 2x Dreamtaq™ green PCR master mix (Fermentas).

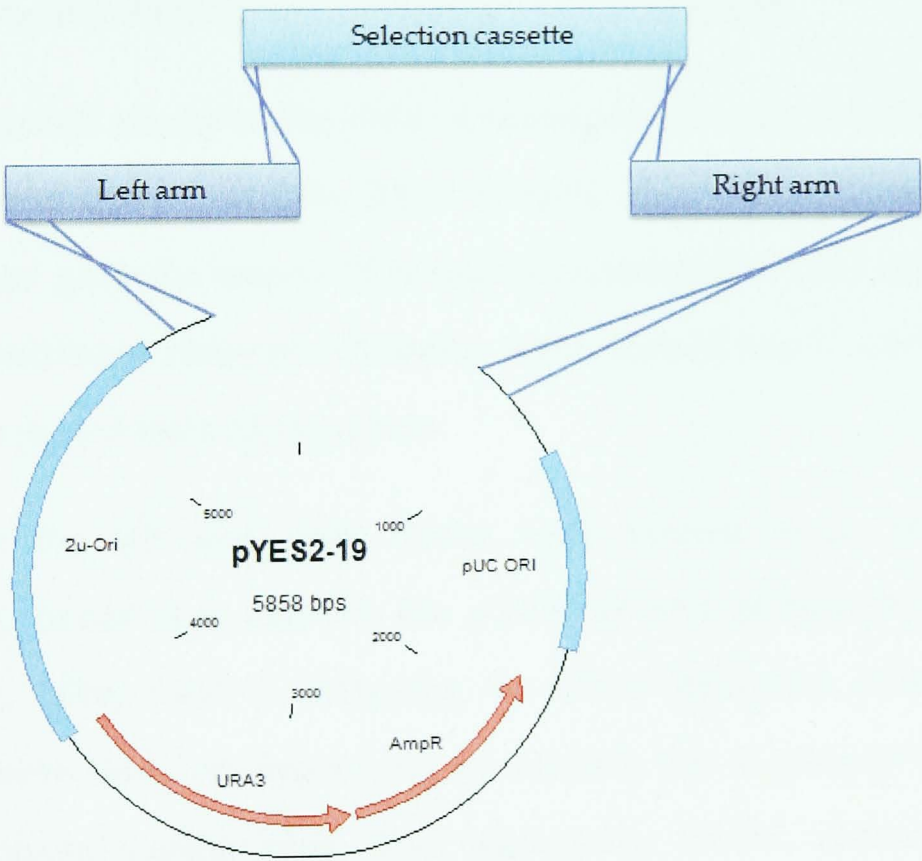


Figure 3.3 Schematic outlining construction of Dicer disruption plasmids. A complete plasmid was made by homologous recombination of the three fragments to the linearised plasmid (pYES2-19). Additional sequences were added to the left and right arms to allow homology to the linearised plasmid and selection cassette.

The third fragment was a hygromycin cassette from the pOlihygtrpc plasmid (Patel *et al.*, 2008) that contained the *hph* gene with an *oliC* promoter and *trpC* terminator from *A. nidulans*, in a plasmid reported to be based on pBluescript SK+. PCR amplification of the cassette used primers just outside the hygromycin cassette and the Phusion High-Fidelity polymerase that possesses a proof-reading activity to minimise mis-incorporation errors.

Before use in yeast homologous recombination, the PCR products of the left and right arms were cloned into pGEMT easy (Promega, UK) and transformed into *E. coli*. The recovered plasmids were sequenced over the insertion sites and found to be correct. The three fragments and the linearised vector for each Dicer disruption plasmid were transformed into yeast and uracil independent transformants were selected.

Despite repeated attempts, the yield of transformants was never in excess of 20 - 30 colonies (rather than the 500 + typically obtained in this method) and none of these gave the colony PCR results expected had they contained the correct recombinant plasmids. Plasmids were rescued into *E. coli* but did not show the expected restriction pattern.

Given that the left and right flanks were known to be correct from sequencing, doubt was cast on the pYES2-19 and pOlihygtrpc plasmids (Patel *et al.*, 2008) used in designing the Dicer disruption plasmids. Both plasmids were sent for sequencing to confirm the sequences around the intended recombination sites. The sequencing results showed that the pYES2-19 plasmid was correct (Appendix 2). However, the pOlihygtrpc sequence showed this plasmid was based on pBluescript (KS) not pBluescript (SK) as previously thought and this was sufficient to prevent the recombination process (Appendix 2). The cloning strategy was redesigned in

light of this information. Using the correct sequences, the revised fragments were PCR amplified as before (Fig 3-4).

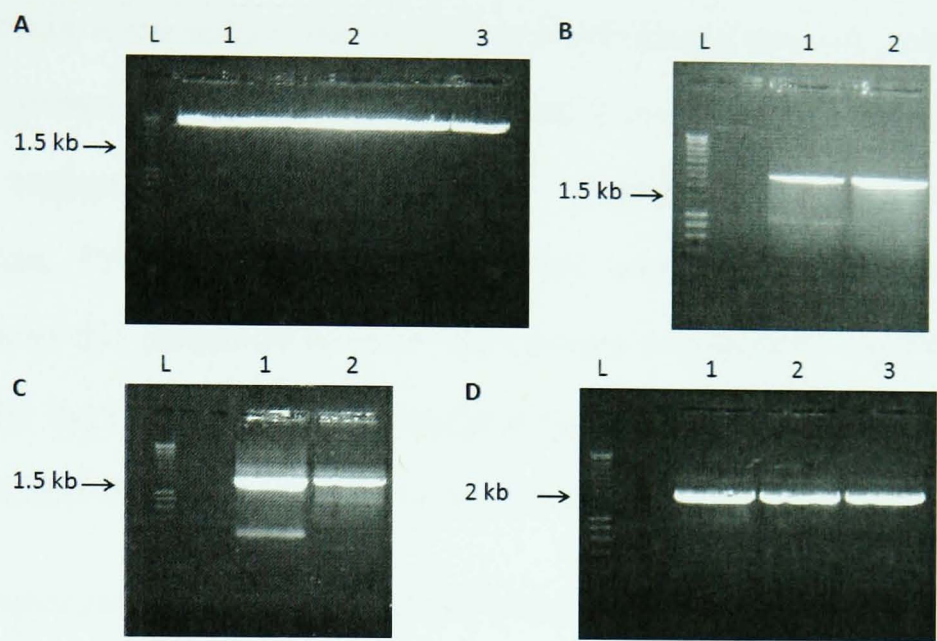


Figure 3.4 Examples of PCR amplified DNA fragments used for the construction of Dicer disruption plasmids. A: 1-3 linearised pYES2-19 (vector), B: 1-2 hygromycin cassette, C: 1.5 kb left and right arms for pYES2-D2, D: 2 kb left and right arms for pXMAS-D2.

The fragments were ligated into a pGEMT vector, transformed into and rescued from *E. coli*, and sent for sequencing. The sequencing results for the arms and hygromycin cassette were BLAST analysed against the *Botrytis* online genome database and the new pOlihygtrpc map sequence and found to be correct. The pYES2-19 and new pOlihygtrpc map are shown in Appendix 2.

Yeast transformations using the three fragments and linearised pYES2-19 vector produced over 500 yeast colonies for each Dicer disruption plasmid while the linearised pYES2-19 (vector-only control) transformation gave no colonies after two days growth on YSDOM selective media. Twenty yeast colonies for each plasmid were subjected to colony PCR using primers previously used to amplify the fragments. All twenty yeast colonies for each of the three plasmids showed the presence of the left arm, right arm and

hygromycin cassette. One colony for each plasmid was rescued into *E. coli* for further propagation and analysis.

The plasmids were subjected to PCR analysis using several primer sets (Fig 3.5). The primer sets respectively B, C and E confirmed presence of the two flanking regions and the hygromycin cassette, but gave no positional information. Primer sets A and D were used to confirm whether the fragments in the plasmids were in the correct orientation. All PCR reactions yielded the expected band sizes suggesting that the recombination had been successful and that the plasmids were correctly made (Fig 3.6).

Another approach to confirm that the Dicer disruption plasmids were correct was by digestion using restriction enzymes. The correct plasmid, after digestion with selected enzymes, would produce bands of predicted sizes. The restriction enzymes used were *Eco*RI and *Hind*III for pYES2-D1 (6.16 kb, 4.1 kb and 0.82 kb), *Xba*I for pYES2-D2 (5.94 kb, 4.17 kb and 1.04 kb) and *Hind*III and *Sac*II for pXMAS-D2 (6.67 kb and 5.35 kb). Digestion for all three plasmids gave these predicted bands as shown in figure 3.7. This analysis confirmed the presence of the selected restriction sites and correct construction for both Dicer disruption plasmids. Plasmids were also sequenced across recombination sites using appropriate primers. This again confirmed the correct formation of the three plasmids by in yeast recombination.

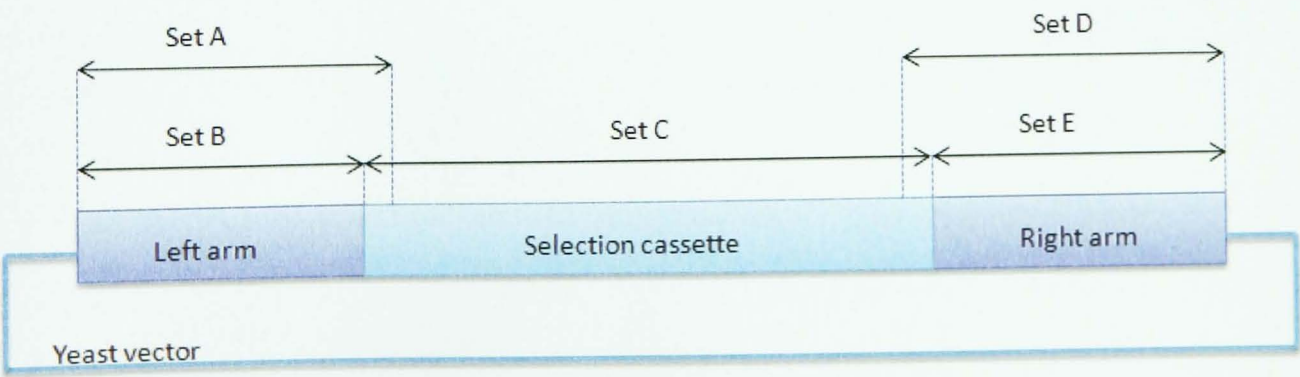


Figure 3.5 The PCR primer sets A, B, C, D and E were used to confirm correct construction of the Dicer disruption plasmids. Primers used are in appendix 1. Primer sets B, C and E checked for the left arm, selection cassette and right arm. Primer set A and D were designed to confirm correct recombination between the arms and selection cassette.

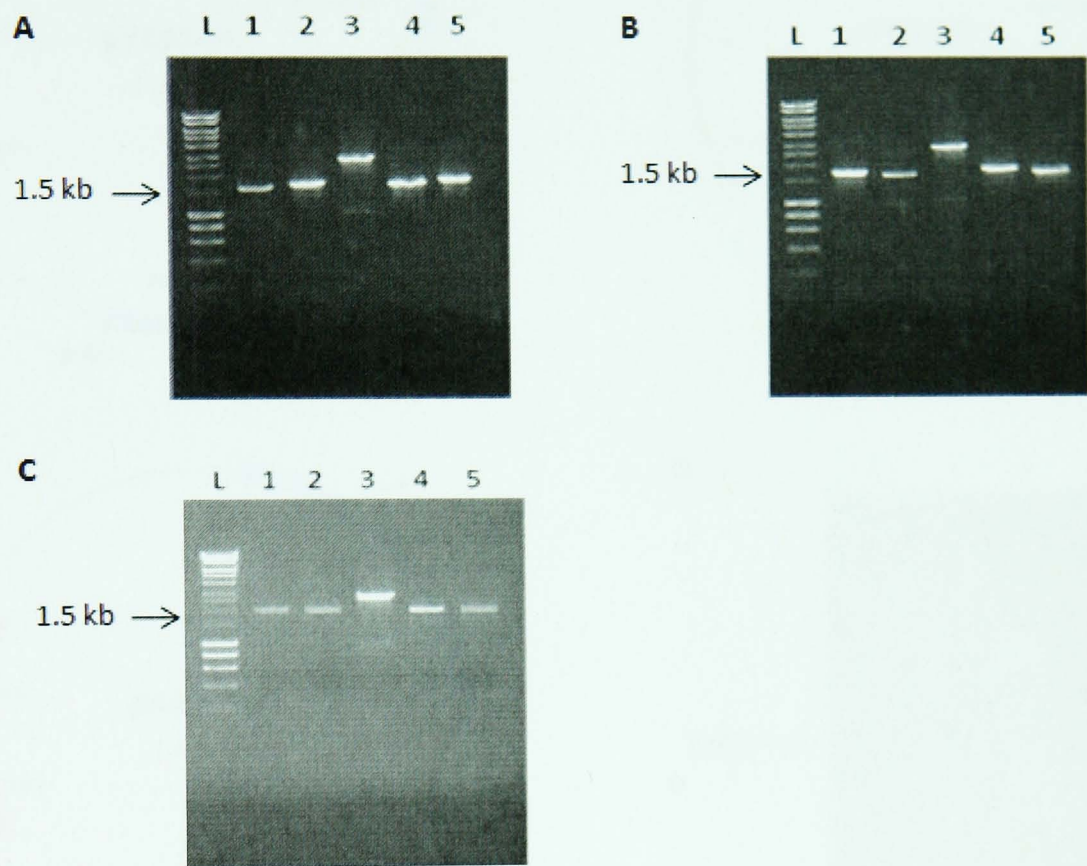


Figure 3.6 PCR analyses to confirm correct construction of pYES2-D1, pYES2-D2 and pXMAS-D2 using primer sets illustrated. In all cases the expected sizes were obtained. A: pYES2-D1. 1: primer set B (1.5 kb), 2: primer set E (1.5 kb) 3: primer set D (2.7 kb), 4: primer set A (1.6 kb), 5: primer set D (1.6 kb). B: pYES2-D2. 1: primer set B (1.5 kb), 2: primer set E (1.5 kb), 3: primer set D (2.7 kb), 4: primer set A (1.69 kb), 5: primer set D (1.5 kb). C: pXMAS-D2. 1: primer set B (2 kb), 2: primer set E (2 kb), 3: primer set D (2.7 kb), 4: primer set A (2.1 kb), 5: primer set D (2.1 kb).

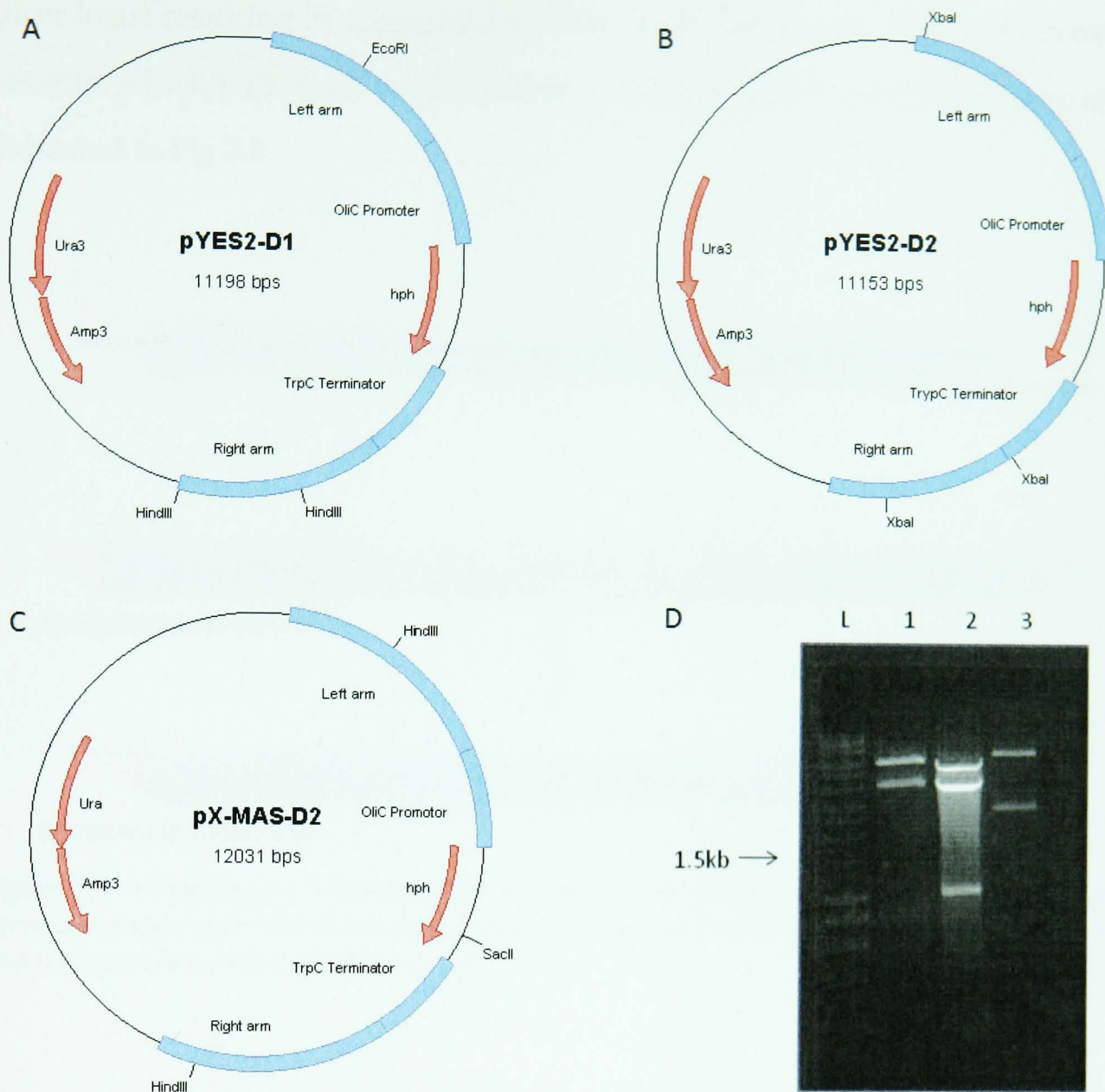


Figure 3.7 Schematics and restriction digest patterns for Dicer disruption plasmids. Selected restriction enzyme sites are shown in schematics (A, B, C) and digested patterns are shown (D) 1; pYES2-D1 was digested with *EcoRI* and *HindIII* (6.16 kb, 4.1 kb and 0.82 kb), 2; pYES2-D2 was digested with *XbaI* (5.94 kb, 4.17 kb and 1.04 kb) and 3; pXMAS-D2 was digested with *HindIII* and *SacII* (6.67 kb and 5.35 kb).

3.3.3 Attempted disruption of *Dcr1* and *Dcr2*

All of the above Dicer disruption plasmids were designed so that amongst a population of transformants, there should be homologous recombination between the regions flanking hygromycin resistance cassette and the desired

Dicer locus resulting in replacement of the native gene with the hygromycin cassette, which will result in deletion of a central portion of the Dicer gene as indicated in Fig 3.8.

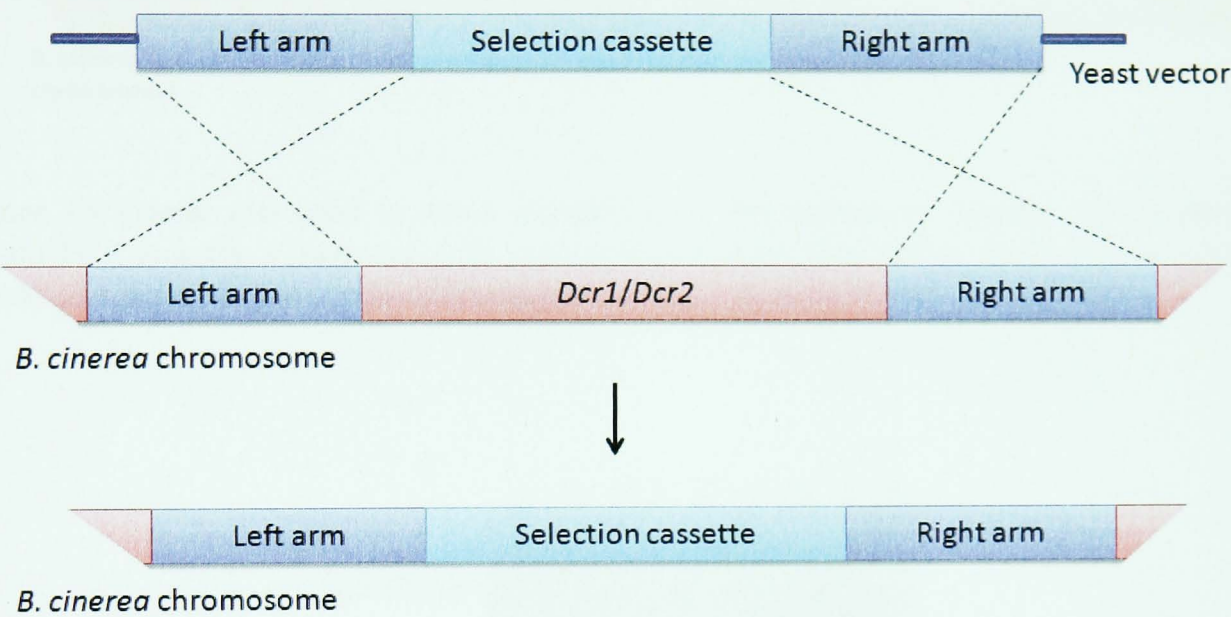


Figure 3.8 Homologous recombination between the cloned flanking section and the corresponding chromosomal location would result in removal or deletion of the Dicer gene and incorporation of the selection cassette.

3.3.3.1 Transformations with pYES2-D1, pYES2-D2 and pXMAS-D2

After several successful *B. cinerea* transformations with pYES2-D1, pYES2-D2 and pXMAS-D2, a total of 180, 100 and 100 *B. cinerea* transformants were obtained.

Transformants using pYES2-D1 were analysed by PCR using primer set F (Fig 3.9) that would amplify a 1.6 kb fragment when the disruption cassette had integrated into the correct locus, and primer set G that would identify the presence of wild type *Dcr1*. Primer set F and G identified 12 transformants as possible *Dcr1* mutants from 180 transformants (Fig 3.10).

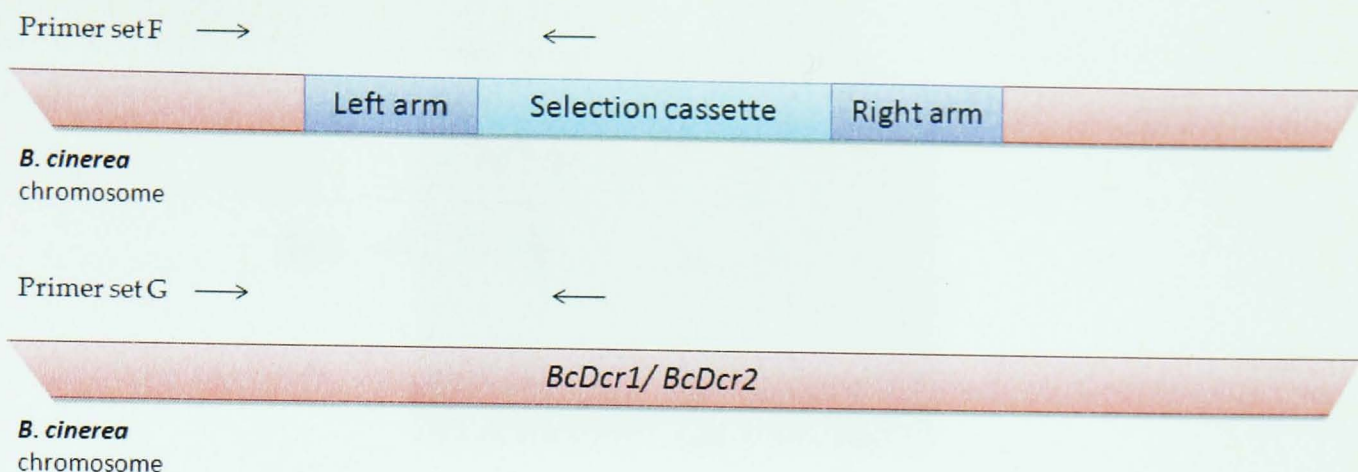


Figure 3.9 Primer sets used to check integration of the disruption cassette. Primer set F would PCR amplify a fragment with correct homologous integration, while primer set G would identify an undisrupted chromosome.



Figure 3.10 Screening for *Dcr1* disrupted transformants by PCR using primer set F. The electrophoresis gel picture shows identification of possible *Dcr1* disrupted transformants as indicated by presence of a 1.6 kb fragment in a small proportion of the transformants. L- Hyperladder; lane 4, 6 and 8- possible *Dcr1* disrupted transformants.

Southern blot analysis on the 12 possible *Dcr1* disrupted lines, *B. cinerea* B05-10 wild type and 3 non-targeted transformants used as controls were prepared using *EcoRI* digested DNA. The probe was a *Dcr1* fragment that would anneal to *Dcr1* when present. As seen in Fig 3.11, the probe annealed to 4 kb DNA fragments for all lines tested, including all the possible *Dcr1* mutant lines. This size band is indicative of a wild-type *Dcr1* locus showing that none were the desired knockout. A *Dcr1* knockout allele would give a 2 kb size band.

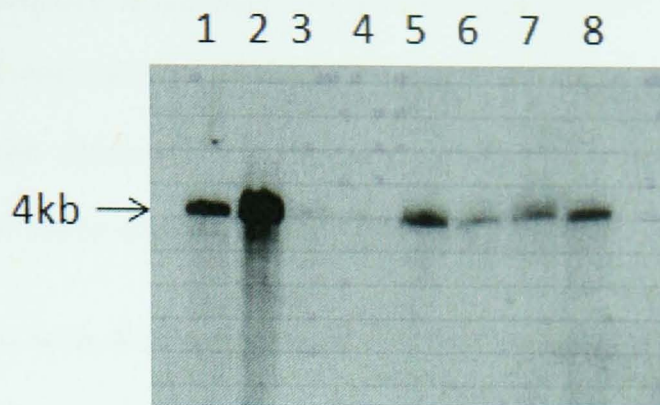


Figure 3.11 Southern analysis of potential *Dcr1* disrupted strains. *EcoRI* digested DNAs were probed with a labelled fragment of *Dcr1*. The transformants were not disrupted since the hybridized signal was apparent in all lanes which showed that *Dcr1* was still present in the genome DNA. Weak signal in lanes 3 and 4 due to lower amounts of genomic DNA. 1: *B. cinerea* wild type (control); 2-8 transformants.

PCR analysis on the 200 transformants obtained using pYES2-D2 and pXMAS-D2 with primer set G (Fig 3-8) showed that all the transformants contained wild-type *Dcr2* (3-12), suggesting gene disruption had not occurred in any of these transformants.



3.12 Screening of *B. cinerea* lines transformed with *Dcr2* disruption plasmids. Results show that using primer set G which detects the *Dcr2* gene, none of the lines were positive for *Dcr2* disruption. L- Hyperladder; 1- B05-10 control; 2 – 10 possible *Dcr2* mutants.

To improve the chances of knockout being generated during transformation, the transformation procedure was modified. One approach used Dicer disruption cassettes digested away from the plasmid vectors, as a linearised

fragment should require a double recombination event to incorporate the marker into the chromosome. The enzymes *SpeI* and *ApaI* were used to cleave out the Dicer disruption cassettes from pYES-2-D1 and pYES2-D2, while *KpnI* and *SpeI* were used for pXMAS-D2.

The other approach was the split marker gene disruption method (Choquer *et al.*, 2008) which used two truncated overlapping fragments of the replacement cassettes instead of a complete cassette. The two truncated cassettes were PCR amplified from the Dicer disruption plasmids. Truncated cassettes from pYES2-D1 and pXMAS-D2 were made for the disruption of *Dcr1* and *Dcr2*, respectively. The first truncated cassette carried the Dicer left arm and the hygromycin cassette truncated at its 3' end. The second truncated cassette carried the same hygromycin cassette truncated at its 5' end and the Dicer right arm. The truncated cassettes shared a 100 bp overlapping sequence within the hygromycin gene. The truncated cassettes at a concentration of 50 µg ml⁻¹ DNA each was used for *B. cinerea* transformation. Plasmids pYES2-D1 and pXMAS-D2 were used as control.

Despite several transformations no hygromycin resistant colonies were found for either approach, while the control plasmids produced *ca.* 10 hygromycin resistant colonies for each transformation.

3.3.4 Attempted gene disruption in a ku70 background

The transformation attempts listed above had failed to give a targeted knockout despite screening several hundred transformants. This suggested that either disruption of *Dcr1* or *Dcr2* was detrimental to the fungus, or that homologous recombination at these loci is abnormally low. During the

course of this work it was reported that mutant strains of fungi lacking either the *ku70* or *ku80* genes, involved in non-homologous end joining, were far more proficient recipients for gene targeting. A *ku70* mutant of *B. cinerea* was kindly provided by M. Choquer (Choquer *et al.*, 2008).

The *B. cinerea* *ku70* strain was already hygromycin resistant as this marker had been used to disrupt the *ku70* gene, therefore new dicer disruption plasmids had to be made with an alternative selectable marker. Nourseothricin was chosen as the selective marker for the new plasmid because this has already been used successfully within our laboratory for transformation of *B. cinerea* (Patel *et al.*, 2010). Priority for plasmid construction was placed on a *Dcr2* disruption plasmid before constructing a *Dcr1* disruption plasmid. This was to determine the efficiency of transformation with the *Botrytis* *ku70* strain before the second plasmid would be made. Also, it has been reported in other filamentous fungi that of the two Dicer genes present, Dicer 2 played an important role in RNA silencing (Segers *et al.*, 2007; Kadotani *et al.*, 2004).

For construction of the *Dcr2* disruption plasmid having nourseothricin resistance selection, the use of the existing Dicer flanking fragments and linearised pYES2-19 vector in pXMAS-D2 was suitable but required a nourseothricin cassette fragment. The 2 kb nourseothricin cassette having an *A. nidulans* promoter (*oliC*) and terminator (*trpC*) and nourseothricin gene (*nat1*) was PCR amplified from pNR2 (Kuck and Hoff, 2006) using specific primers (Appendix 1) and a Phusion High-Fidelity polymerase PCR kit.

For the construction of the nourseothricin resistant *Dcr2* disruption plasmid, yeast recombinational cloning using the three PCR amplified fragments and linearised vector produced over 500 yeast colonies after two days growth on

YSDOM selective media. Colony PCR on 20 yeast colonies gave expected bands for the presence of the three fragments. A plasmid from one colony was selected and rescued into *E. coli* and designated pNDT-2. To confirm that pNDT-2 was correct as designed, the plasmid was subjected to PCR analysis using primer sets A, B, C, D and E (Fig 3.4). Results showed that the expected constructs were present and in the correct orientation (Fig 3.13).



Figure 3.13 PCR bands confirming correct construction of pNDT-2. Expected bands were produced when using primer sets fig 3.4; 1: primer set B (2 kb), 2: primer set C (2 kb), 3: primer set E (2.1 kb), 4: primer set A (2.1 kb) and 5: primer set D (2.1 kb).

The next approach to confirm that pNDT-2 was correct was by using restriction analysis. This was the use of restriction enzymes sites on the plasmid. The plasmid was digested with *Bgl*II and *Hind*III and gave expected size bands (6.4 kb, 3.9 kb, 0.65 kb and 0.19 kb; Fig 3-14). This analysis confirmed the presence of the selected restriction sites and confirmed the correct construction of pNDT-2.

The final approach taken to confirm if the plasmid was correct was to sequence the plasmid over the recombination sites. Results from sequencing showed the pNDT-2 map was as expected.

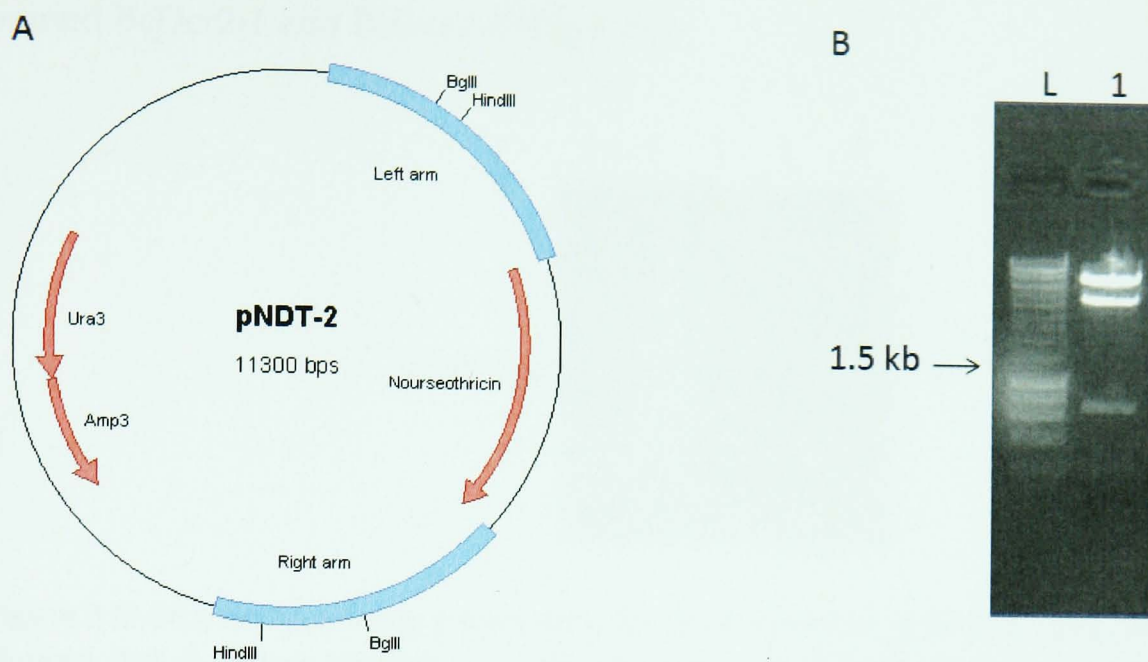


Figure 3.14 Plasmid map and restriction pattern for pNDT-2. A; Schematic shows selected restriction enzyme sites and, B; digestion using *Bgl*III and *Hind*III which gave expected sizes (6.4 kb, 3.9 kb, 0.65 kb and 0.19 kb).

3.3.5 *B. cinerea* ku70 transformations

The Dicer disruption plasmid pNDT-2 was transformed into *B. cinerea* ku70.

After 40 independent transformations just 20 transformants were obtained having nourseothricin resistance on media containing 50 μg nourseothricin ml^{-1} . The transformants were subcultured 3 times on 100 μg nourseothricin ml^{-1} MEA media to remove the risk of heterokaryon survival.

Genomic DNA was extracted from each transformant and PCR analysis was performed using primers to investigate the presence of *Dcr2* including the ku70 recipient strain as control. PCR results showed that the positive controls and all but two of the transformants produced PCR products corresponding to the native *Dcr2*. Whilst all were positive for β -tubulin, this

showed that there were two possible candidates as *Dcr2* mutants which were named BcDcr2-1 and BcDcr2-2 (Fig 3.15).



Figure 3.15 PCR analysis using primer set G for of the presence of the *Dcr2* gene. The results showed that *B. cinerea* mutants (1, 2) lacking native *Dcr2* while the ku70 (3) control was positive for *Dcr2*.

Southern analysis was carried out on BcDcr2-1 and BcDcr2-2 as described in Methods and Materials using *SphI* to digest the DNA. Two probes were used, probe A was situated to the left side of the integrated cassette, while probe B was situated on the right (Fig 3.16).

SphI was selected because it would digest once within the nourseothricin cassette and at sites in the genomic DNA outside the targeting cassette, allowing both recombination sites to be analyzed. If targeting was successful, a *Dcr2* mutant would give expected band size of 4.9 kb for probe A and 10.9 kb for probe B while the wild type would be 15.9 kb for each.

Results from Southern analysis showed that probe A hybridised to the two mutants and control *B. cinerea* ku70 with a 4.9 kb fragment for the mutants and 15.9 kb fragment for the control (Fig 3.17). For probe B, the probe hybridised to a 10.9 kb fragment for both the mutants and 15.9 kb fragment size for the control, indicating successful disruption in both transformants.

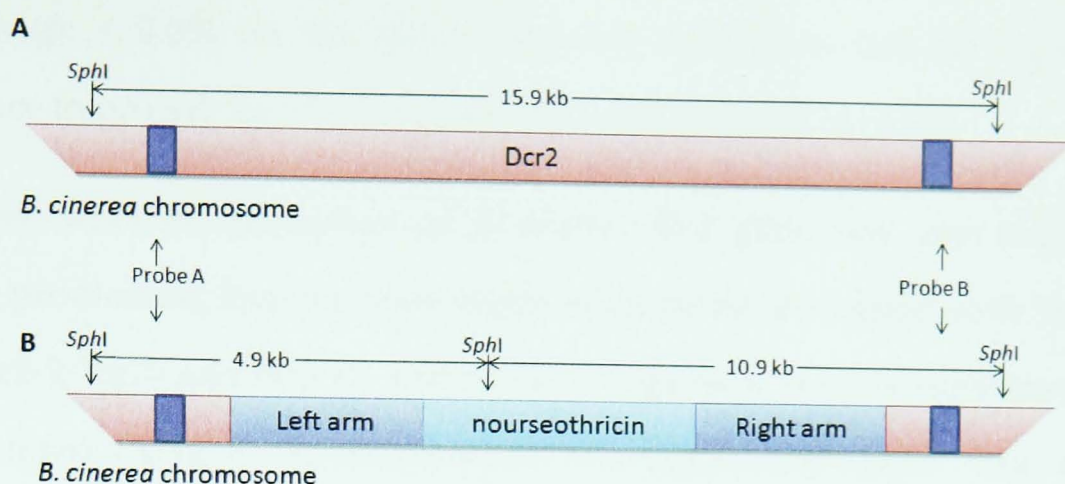


Figure 3.16 Schematic location of probes A and B and cleavage sites for restriction enzyme (*SphI*) used in Southern analysis for *B. cinerea* wild type (A) and *Dcr2* mutants (B).



Figure 3.17 Southern analysis to confirm *Dcr2* disruption. Autoradiographs of *SphI* digested DNA of BcDcr2-1(1), BcDcr2-2 (2) and ku70 (3) probed with probe A (panel A) and probe B (panel B) showing that in both cases the disrupted mutants have a single hybridising band and as predicted these are of a different size to the wild-type signal.

3.3.6 Growth and morphology on *Dcr2* mutants

To determine whether gene disruption for the *B. cinerea* *Dcr2* mutants had any effect on growth rates as has previously been observed in MDL-2 disrupted mutants of *M. oryzae* (Kadotani *et al.*, 2004), growth assays on MEA plates were carried out with *B. cinerea* B05-10 and ku70 as controls in triplicates and radial growth measured after 5 days (Fig 3.18). Statistical

analysis ($p: < 0.05$) on the results showed that there was no significant difference in growth for the *B. cinerea* *Dcr2* mutants and controls.

To assess whether disruption of *B. cinerea* *Dc2* gene had any impact on conidia production, five replicate plates were point inoculated with BcDcr2-1, BcDcr2-2, ku70 and B05-10. After 7 days of growth, conidia were harvested and counted using a haemocytometer and light microscope. The results showed that BcDcr2-1, BcDcr2-2, ku70 and B05-10 had $40, 32, 27$ and 31×10^6 conidia per plate respectively (Fig 3.19). Statistical analysis (Student *t*-test) showed that there was a significant difference ($p: < 0.05$) in conidia production between the different line. This showed that the *B. cinerea* *Dcr2* mutants had an 8 % increase in conidiation compared to the *B. cinerea* ku70 control parent strain. The B05-10 conidia population was also determined and included as a reference in Fig 3.18.

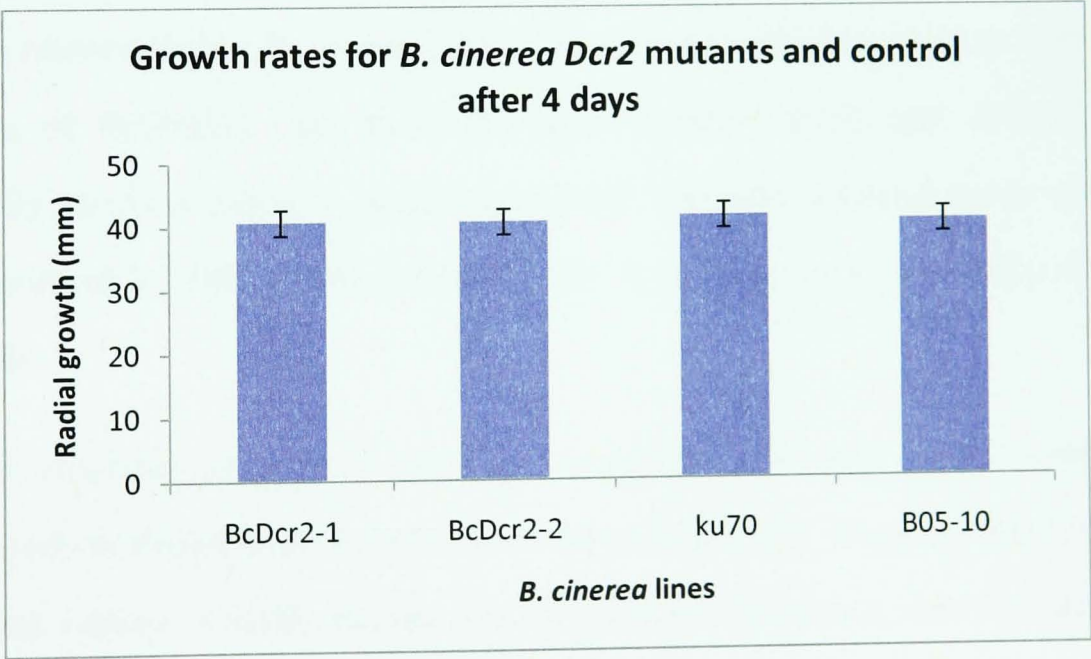


Figure 3.18 Growth rates for *B. cinerea* *Dcr2* mutants (BcDcr2-1, BcDcr2-2) and controls (ku70, B05-10) The *B. cinerea* lines were point inoculated onto MEA plates and grown for 4 days. Statistical analysis ($p: < 0.05$) for *Dcr2* mutants and controls showed no significant difference in radial growth rates.

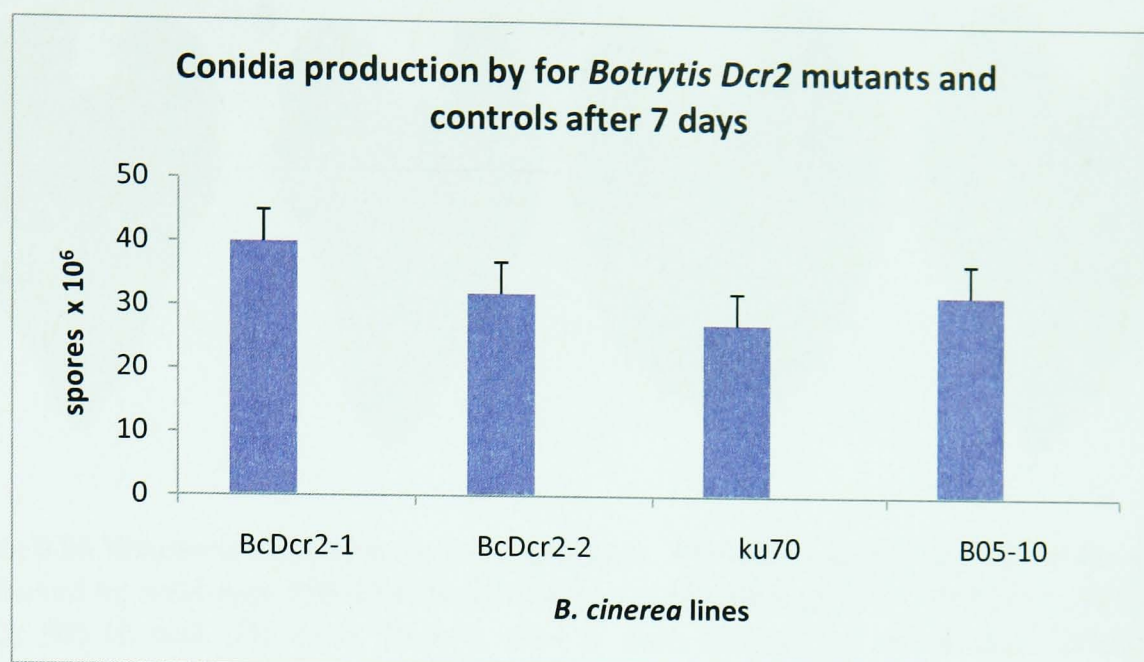


Figure 3.19 Conidia production by *B. cinerea* *Dcr2* mutants. After 7 days of growth, data showed that BcDcr2-1, BcDcr2-2, ku70 and B05-10 had 40, 32, 27 and 31 x 10⁶ conidia per plate respectively. There was a significant difference in conidia production between the lines. This shows that both *Dcr2* mutants had more than 8 % increase in conidia populations compared to the parent ku70 strain. *B. cinerea* B05-10 was also determined and included as a reference.

Also, to determine whether *B. cinerea* *Dcr2* mutants had malformed conidia as was observed for *M. oryzae* *Dicer* one mutants (Kadotani *et al.*, 2004), the conidia of BcDcr2-1 and BcDcr2-2 and controls ku70 and B05-10 were carefully studied using a light microscope. Results showed there were no distinguishable differences between the *B. cinerea* *Dcr2* mutants and the controls.

Fungal virulence assays of the *Dcr2* mutants, ku70 and B05-10 were also measured on dwarf bean leaves after 4 days (Fig 3.20). The statistical analysis (Student *t*-testp: < 0.05) on the lesion growth diameters showed that that there was no significant difference between the *B. cinerea* *Dcr2* mutants, ku70 and B05-10 (Fig 3.21).

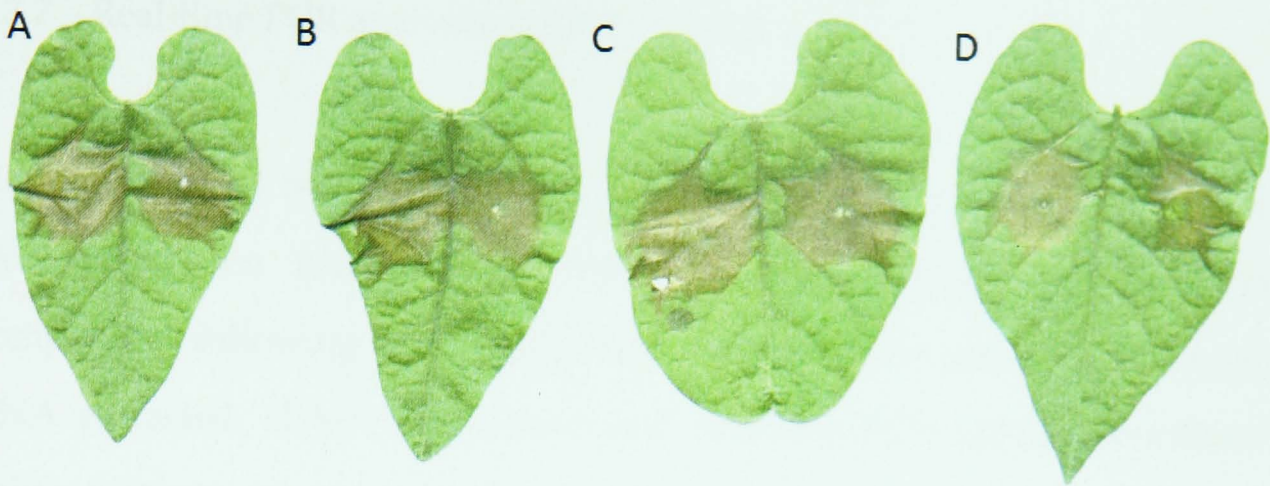


Figure 3.20 Virulence assays on dwarf bean leaves. All lesions on the left side of the mid rib are caused by wild-type B05-10 with the lesions on the right from (A) BcDcr2-1, (B) BcDcr2-1, (C) B05-10 and (D) ku70. Results after 4 days showed no observable difference in virulence between B05-10 and *Dcr2* mutants.

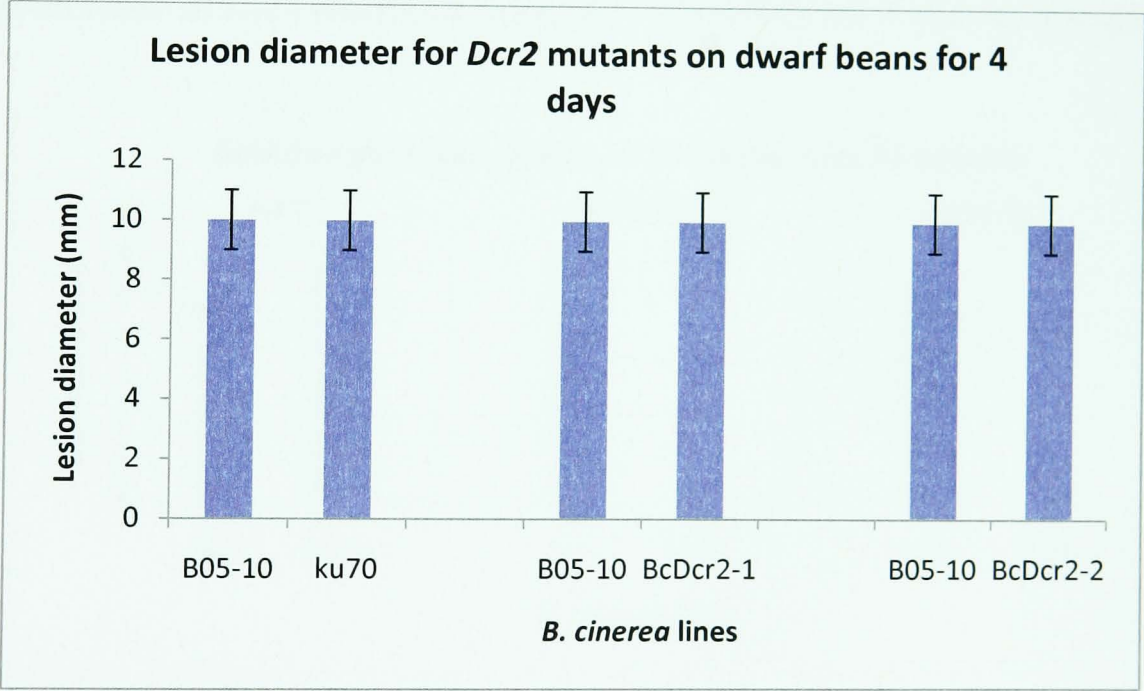


Figure 3.21 Lesion diameters for *B. cinerea* *Dcr2* mutants, ku70 and B05-10 as control on dwarf bean leaves after 4 days. There was no significant difference in virulence for the *Dcr2* mutants and controls.

3.3.7 Real-time PCR analysis on *Dcr2* mutants

Having obtained two *Dcr2* mutants, it was desirable to confirm the lack of *Dcr2* expression and whether *Dcr1* had altered expression levels to compensate following *Dcr2* disruption. Cultures were grown for 7 days, RNA extracted, cDNA synthesized and real-time PCR (qPCR) performed using primers sets to quantify *Dcr1* and *Dcr2* expression along with β -tubulin (NCBI accession number : U27198).

Using qPCR, the expression levels for *Dcr1* and *Dcr2* genes for BcDcr2-1, BcDcr2-2 and ku70 (control) were collected and analysed by relative expression to β -tubulin. The relative expression levels (Fig 3.22) showed that although *Dcr2* was no longer expressed in the *Dcr2* mutants, there was a small increase in *Dcr1* levels for BcDcr2-1, while BcDcr2-2 was unchanged.

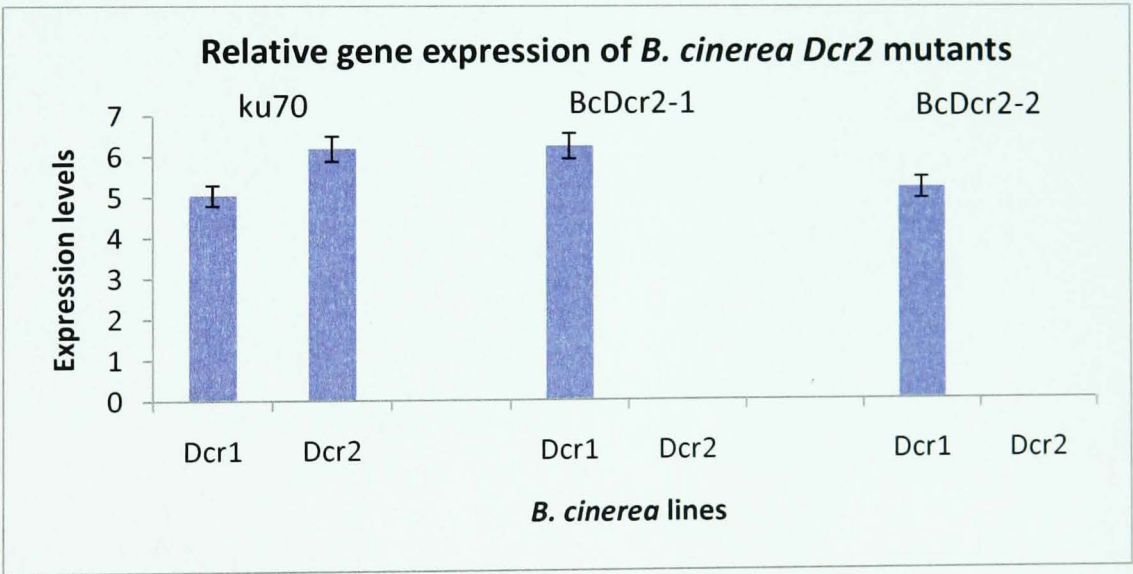


Figure 3.22 qPCR relative expression levels for *B. cinerea* *Dcr2* mutants (BcDcr2-1 and BcDcr2-2) and ku70 (control) showing that *Dcr2* transcripts were absent in the disrupted mutants and that *Dcr1* levels showed no consistent change.

The *B. cinerea* *Dcr2* mutants BcDcr2-1 and BcDcr2-2 have been subjected to PCR analysis, Southern analysis and qPCR, and all these confirmed the loss

of *Dcr2*. Although there was a small increase in conidiation for the BcDcr2-1 and BcDcr2-2 compared to *B. cinerea* ku70, the *B. cinerea* *Dcr2* mutants showed no other distinguishable change in morphology, growth rate and virulence. *Dcr1* expression levels do not appear to be increased to compensate for disruption of *Dcr2*.

3.4 Discussion

3.4.1 Bioinformatics

Using BLAST search with DICER protein sequences from selected *Ascomycete* fungi, two Dicer genes were identified in *B. cinerea*. The protein sequences for *B. cinerea* DICER 1 and 2 are typical of fungal dicers, with the expected motifs, such as the Deadbox, Helicase, duf83 and RNase -a and -b, and each gene had three conserved introns which was a common characteristic for the DICER proteins (Carmel and Hannon, 2004; Soifer *et al.*, 2008).

Several of the protein sequences and alignments for some fungal DICER proteins had to be corrected before use. This shows that not all the fungal protein sequences available on the databases were correctly annotated and that careful assessment of the protein sequences needs to be made before use in molecular experimentation and analysis.

Phylogenetic analysis of both *B. cinerea* DICERs and those from selected fungi showed that the DICER proteins sequences of *B. cinerea* were closely related to equivalents in *S. sclerotiorum*, *N. crassa* and *M. oryzae*. *B. cinerea* DICERs showed a highest protein sequence identity to *S. sclerotiorum* reflecting the close taxonomic relationship between these species. The *Ascomycete* Dicers grouped together with the animal Dicers while the plant Dicers formed their own clade as expected, given the closer relationship between fungi and animals (Gupta, 1995).

As is typical for most *Ascomycetes*, the bioinformatic analysis clearly shows that *B. cinerea* has two Dicer genes. From this analysis it was not possible to determine whether RNA silencing in *B. cinerea* would be analogous to *N.*

crassa, where both Dicer genes contributed to RNA silencing or be like *M. oryzae*, *A. nidulans* and *C. parasitica* where only the DICER 2 protein was important for RNA silencing, which is why a disruption approach was taken to investigate these genes.

3.4.2 Dicer disruption constructs

To create the *B. cinerea* dicer disruption cassettes, the initial option was the use of restriction enzymes, but this was abandoned when no appropriate restriction sites were found. An alternative approach was to create the plasmids by in-yeast recombination.

Many gene disruption cassettes have been used in *B. cinerea*. The length of the flanking regions for the targeted genes carried by the cassettes have been diverse, but generally, flanking regions would be between 400 - 2000 bp (Choquer *et al.*, 2008; Tudzynski and Siewers, 2004; Siewers *et al.*, 2004; Viaud *et al.*, 2003; ten Have *et al.*, 1998). For this work, 1500 and 2000 bp flanking regions were made in an attempt to increase the chances of homologous recombination. Although the frequency of targeted disruption depends on the targeted gene and the recipient host, reports have shown that the efficiency of targeted gene disruption among *B. cinerea* transformants is usually higher than 50 % (Tudzynski and Siewers, 2004). An example would be the disruption of the *B. cinerea* gene *cel5A* that codes for endo- β -1, 4-glucanase which had target efficiency of 60 % (Noda *et al.*, 2007). But there have occasionally been cases of low to null target efficiency, as reported for the *B. cinerea* *Cnd15*, a putative sesquiterpene cyclase-encoding gene (Choquer *et al.*, 2008).

Transformation with the complete Dicer disruption plasmids generated no targeted disruption of *Dcr1* or *Dcr2* in *B. cinerea* B05-10 despite screening over 100 transformants for each. Given that disruption of the Dicer genes in other filamentous fungi was not lethal, the lack of Dicer disruption was most likely to be low targeting efficiency rather than any detrimental affect from gene disruption. The ongoing low transformation efficiency may have impacted on Dicer gene disruption attempts using the linearised overlapping constructs.

There were contradictory results from PCR analysis which identified possible *B. cinerea Dcr1* mutants that could not be confirmed by Southern analysis. Either there were false positives or one possible explanation for this would be that the *B. cinerea Dcr1* transformants identified by PCR analysis were not pure cultures, however, they subsequently lost the disruption events during the subculturing process before Southern analysis.

The *Botrytis ku70* strain was obtained which had inactivation of the non-homologous end joining DNA (NHEJ) repair pathway, therefore increasing the frequency of targeted gene disruption. NHEJ binds broken DNA ends using the bridging activity of the ku complex (Hefferin and Tomkinson, 2005). The ku70/ku80 have also been disrupted in other fungi including *N. crassa* (Ninomiva *et al.*, 2004), *M. grisea* (Villalba *et al.*, 2008), *Aspergillus spp* (Takahashi *et al.*, 2006, Chang, *et al.*, 2010) and *C. neoformans* (Goins *et al.*, 2006). In all cases, this has resulted in a significant increase of homologous integration rates.

For this work, two *Dcr2* mutants were identified by PCR after transformation of ku70 protoplasts with *Dcr2* disruption plasmids (pNDT-2). The mutants were analysed by using two Southern analyses probes to determine double

recombination of the flanking regions. Out of twenty nourseothricin transformants, two *Dcr2* mutants were obtained, giving a transformation efficiency of 10 %, still far lower than reported for other *B. cinerea* genes.

In *M. oryzae*, disruption of MDL-1 and MDL-2 genes resulted in conidia malformation and reduction in mycelial growth respectively. Therefore, the two *B. cinerea Dcr2* mutants were carefully observed for conidia malformation and growth rate compared to *B. cinerea* wild type. In addition, the level of virulence for the mutants on plant leaves was carried out. In all, no obvious difference was observed for the *B. cinerea Dcr2* mutants compared to the controls. However, for conidia production, one of the the *B. cinerea Dcr2* mutants displayed an 8% increase in conidia compared to *B. cinerea ku70* control, however the other was normal. This probably reflects variation within the experiment and is unlikely to be significant. It is not uncommon to have changes in morphology with loss of a Dicer gene such as reduction in hyphal growth reported for *Mucor sp* (Nicolas *et al.*, 2010) and *M. oryzae* (Kadotani *et al.*, 2004). To our knowledge, this is the first report showing an increase in conidia production associated with loss of *Dcr2*.

Overall, the results from observing the *B. cinerea Dcr2* mutants show that they were broadly similar to *C. parasitica* (Segers *et al.*, 2007) and *N. crassa* (Catalanotto *et al.*, 2004) in so much as there being no observable morphological changes in growth rate and no apparent conidia malformation identified with loss of *Dcr2*.

3.4.3 Future work

Due to time restrictions *Dcr1* mutants could not be made. However, if plans to create *Dcr1* mutants are made, the suggested approach would be to create a *Dcr1* disruption plasmid with a nourseothricin resistance cassette and to have 2 kb or longer left and right arms from sequences flanking the *Dcr1* gene and transformed into *B. cinerea* ku70 strains following the methodology detailed in Methods and Materials.

With the *B. cinerea* *Dcr2* mutants made, the next step was to determine whether the proposed RNA silencing genes (*Dcr1* and *Argo1*) had any compensatory effect in *B. cinerea* due to the loss of *Dcr2*. This was assessed in the following chapters by qPCR.

To show whether *Dcr2* was involved in RNA silencing, an RNA silencing cassette could be transformed into the wild type and *Dcr2* mutants. If RNA silencing is observed in the wild type and not the *Dcr2* mutants, this would show that RNA silencing in *B. cinerea* is similar to the filamentous fungi *N. crassa* and *A. nidulans*.

4 Evaluation of RNA silencing in *B. cinerea* Dcr2 mutants

4.1 Introduction

The identification of RNA silencing in *N. crassa* was the first report in filamentous fungi (Romano and Macino, 1992). Having discovered the process, there was interest in determining the mechanism and this involved isolation of mutants impaired in silencing. *Neurospora crassa* lines which were initially active in RNA silencing of the endogenous *al-1* gene, were exposed to ultraviolet radiation to create mutants that lost RNA silencing activity (Cogoni and Macino, 1997). Screening of the *N. crassa* mutants identified three lines QDE-1, QDE-2 and QDE-3. These mutations were subsequently mapped to genes that code for the RDRP, ARGONAUT and HELICASE proteins respectively (Lee *et al.*, 2010). These proteins have been reported in other *Eukaryotes* to be essential in the RNA silencing mechanism (Carmel and Hannon, 2004; Soifer *et al.*, 2008). It is important to note that no dicer mutants were isolated in this screening.

One of the important components of the RNA silencing mechanism is the DICER protein (Baulcombe, 2002). The involvement in RNA silencing for this RNase type III enzyme was identified by Zamore *et al.*, (2000) while Bernstein *et al.*, (2001) coined the term DICER which the literature now uses. The DICER protein recognises and cleaves aberrant exogenous and endogenous dsRNA molecules into 21 bp siRNA duplexes. One of the strands from the siRNA duplexes are incorporated into RISC which uses the strands as templates for recognition and degradation of aberrant RNA (Bernstein *et al.*, 2001).

The ability to induce RNA silencing has been used to confirm a gene's involvement in RNA silencing (Nakayashiki and Nguyen, 2008). When a putative RNA silencing gene has been disrupted, the next step would be to determine whether the RNA silencing mechanism remains active. This could be done by silencing an endogenous gene or transgene in the mutant. In *M. circinelloides*, an RNA silencing construct encoding the endogenous *carB* gene involved in carotenoid biosynthesis was transformed into a Dicer 2 mutant (Haro *et al.*, 2009) while for *A. oryzae*, an eGFP silencing construct was transformed into the Dicer 2 mutant which already carried the eGFP fluorescence reporter gene (Kadotani *et al.*, 2004). In both cases, there was no reduction in the transcription levels of the targeted genes which meant that loss of Dicer 2 was sufficient to prevent RNA silencing in these fungi.

Active RNA silencing of *bcass1* in *B. cinerea* gives partially auxotrophic growth on media without arginine supplementation (Patel *et al.*, 2010), where reduced growth indicated the strength of silencing. The *bcass1* gene encodes the enzyme that makes argininosuccinate, an intermediate in arginine biosynthesis (Husson *et al.*, 2003). The degree of silencing could therefore be measured by comparison between growth on minimal media with and without arginine to assess whether the Dicer 2-disrupted strains were still able to perform silencing in a normal manner.

4.2 Aims

The Dicer 2 genes in *A. nidulans*, *M. oryzae* and *C. parasitica* have been reported to play an important role in RNA silencing. This work investigated whether disruption of *B. cinerea* *Dcr2* would inactivate the RNA silencing mechanism in *B. cinerea*. Therefore, the aims were to evaluate the *B. cinerea*

Dcr2 mutants to determine whether these are still able to perform silencing and also to investigate any transcriptional impact on other genes in the silencing pathway.

4.3 Results

Two *B. cinerea* *Dcr2* mutants were produced as described in Chapter 3. The next step was to investigate the role of *B. cinerea* *Dcr2* in RNA silencing by functional analysis. Since the *B. cinerea* *Dcr2* mutants had already been transformed to hygromycin and nourseothricin resistance, a new selection marker was required to introduce the RNA silencing plasmid. Transformation to basta resistance had been used in our laboratory on other fungi, but not on *B. cinerea*, so preliminary investigations were performed to evaluate the resistance of *B. cinerea* to basta and the functionality of this transformation marker.

4.3.1 Establishing selection conditions

Botrytis cinerea was cultivated on a range of basta concentrations to identify the level of resistance of *B. cinerea* to the herbicide. Gamborg's media with basta concentrations of 0, 0.1, 0.5, 2, 5, 10, 15, 25, 35, 50, 75, 100 $\mu\text{g ml}^{-1}$ was prepared. The *B. cinerea* strains B05.10 and ku70 were point inoculated onto the plates in triplicate, and radial measurements of the growing mycelia recorded after 5 days (Fig 4.1). Results show that for both strains, no hyphal growth was observed for plates having basta concentrations of 10 $\mu\text{g ml}^{-1}$ and above. This meant that for *B. cinerea* transformation, the working

concentration of 12.5 $\mu\text{g ml}^{-1}$ basta was used for SH regeneration overlay and surviving transformants subcultured on plates having 25 $\mu\text{g ml}^{-1}$ basta to reduce any risk of heterokaryons. No kill-curve was carried out for the *Dcr2* mutants, since it was considered that the mutants would give similar results as *B. cinerea* B05-10 and ku70.

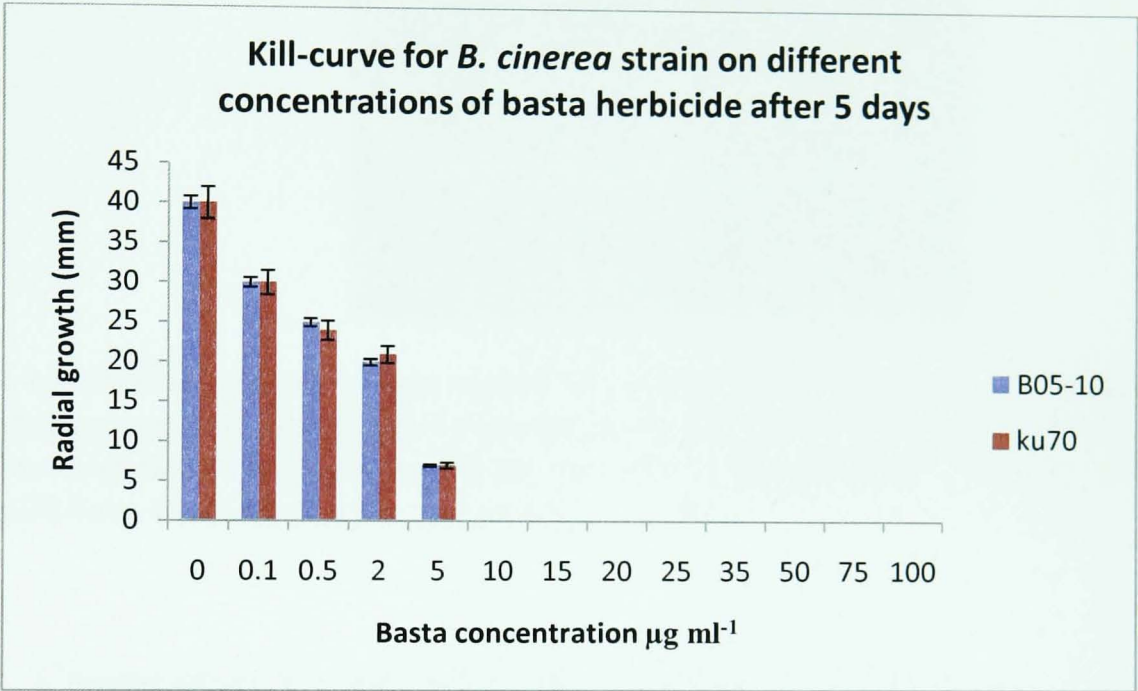


Figure 4.1 Radial growth of *B. cinerea* B05-10 and ku70 after 5 days on Gamborg media supplemented with basta. The results showed that *B. cinerea* hyphal growth was not observed on plates having basta concentrations of 10 $\mu\text{g ml}^{-1}$ and higher.

4.3.2 Transformation of *B. cinerea* to basta resistance

To determine the usability of the basta vector in *B. cinerea* transformations, pCB1530 (Sweigard *et al.*, 1997) with a basta resistance gene (*bar*) controlled by the *N. crassa* glucose-repressible promoter (*grg-1*) (Pall and Brunelli, 1994) was transformed into *B. cinerea* ku70 as described in Materials and Methods, using basta concentration of 25 $\mu\text{g basta ml}^{-1}$. Two independent *B. cinerea* transformations generated a total of 20 basta resistant transformants. The

basta resistant transformant, control DNA pCB1530 and ku70 were subjected to PCR analysis using basta primer set H (Appendix 1) to confirm integration of the basta resistance gene. The PCRs gave the expected size band of 0.54 kb (Fig 4.2). This showed that pCB1530 could be used as a selection marker in *B. cinerea*.

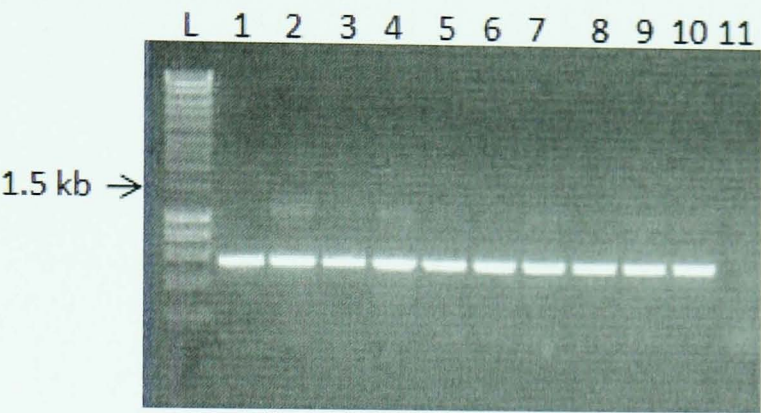


Figure 4.2 PCR analysis using basta primer set H on *B. cinerea* transformants that survived subculturing on basta. Figure shows expected size bands of 0.54 kb on electrophoresis gel to confirm integration of the basta cassette into *B. cinerea*. L: Hyperladder; 1: pCB1530 control; 2–10 (ku70 Basta transformants); 11: parent ku70.

4.3.3 Construction of *bcass1* RNA silencing plasmid (pLOB-bar)

The *bcass1* silencing cassette was derived from pLOB1-MCS which was already reported to cause silencing of the argininosuccinate synthase gene in *B. cinerea* (Patel *et al.*, 2010). This silencing cassette was selected due to the auxotrophic effect that could be scored on plate assays.

The *bcass1* silencing plasmid pLOB-bar was made by excision of the *bcass1* silencing cassette from pLOB1-MCS using restriction enzymes *Hind*III and *Sac*I. The *bcass1* RNA silencing cassette was ligated into the basta resistance vector, pCB1530, which had been linearised using *Hind*III and *Sac*I. The plasmid, designated pLOB-bar, was transformed into *E. coli*.

To confirm whether pLOB-bar was correctly made, PCR amplification was performed using primer set H to detect the presence of the *Bcass1* gene. This gave expected size bands of 0.54 kb on a 1 % electrophoresis gel (Fig 4.3). Restriction analysis using enzymes *Hind*III and *Sac*I gave band sizes of 3.85 kb and 1.68 kb as expected (Fig 4.4). In all, molecular analysis showed that the plasmid was made as designed.

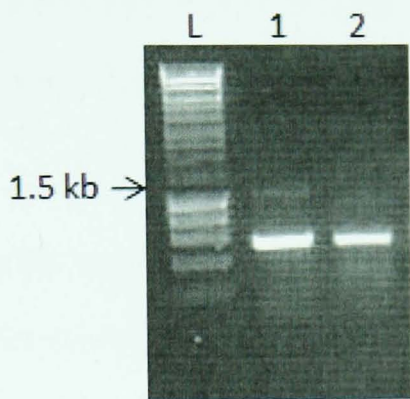


Figure 4.3 PCR analysis on pLOB-bar using *bcast1* primer set H gave an expected size band of 0.54 kb on 1 % electrophoresis gel. This confirms the presence of the partial *B. cinerea bcast1* gene in the plasmid.

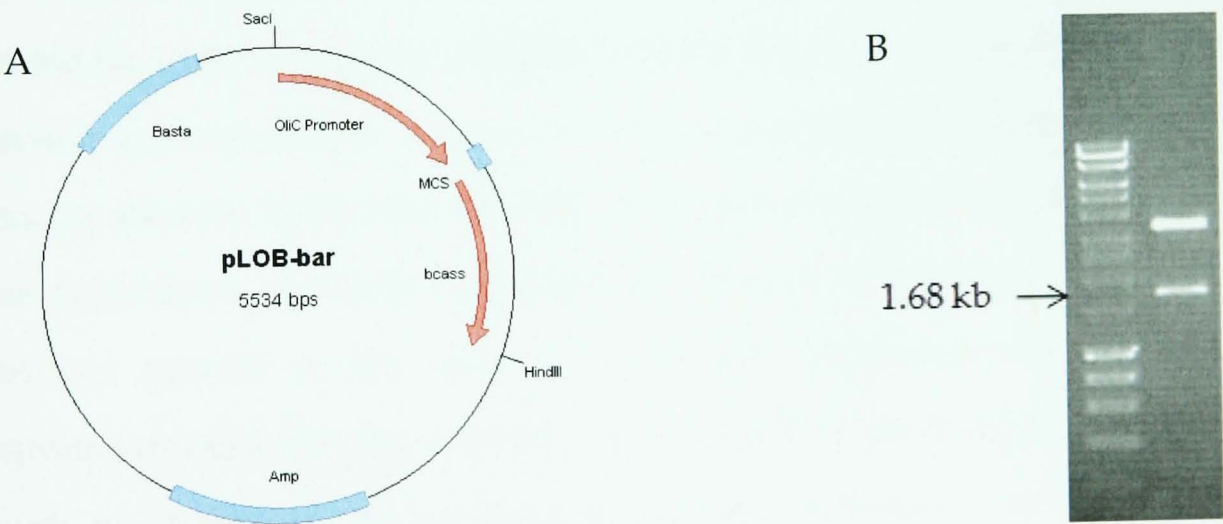


Figure 4.4 A map of the *bcast1* silencing plasmid (pLOB-bar) showing restriction sites (A). Electrophoresis of *Hind*III and *Sac*I digested pLOB-bar giving expected size bands of 3.85 kb and 1.68 kb (B).

4.3.4 *B. cinerea* transformations with pLOB-bar

To assess the RNA silencing ability of the ku70 strain, it was necessary to generate a population of transformants and assess the degree of RNA silencing within a population. Therefore, pLOB-bar was first transformed into the ku70 strain. Transformation was as described in Materials and Methods with the regeneration SH media having arginine supplementation and 12.5 $\mu\text{g ml}^{-1}$ basta overlay. There were 75 basta resistant transformants identified and isolated.

After three subcultures with arginine and 25 $\mu\text{g ml}^{-1}$ basta to purify the transformants, each transformant and the parent ku70 as control was point inoculated onto Gamborg media plates with and without arginine supplementation. The radial growth rates were measured after 5 days growth and data analysed.

A reduction in growth was observed for several of the Sku70 transformants on media without arginine supplementation (Fig 4.5). Figure 4-6 shows that growth was reduced in absence of arginine supplementation by up to 71 % among silenced ku70 lines (Sku70). This demonstrates that the majority of the population of Sku70 transformants showed some *bcass1* silencing by reduced growth in the absence of arginine supplementation while the untransformed ku70 parent strain showed no RNA silencing effect. For this work, any transformant that showed a growth reduction of 10 % or more on media without arginine supplementation was considered to be silenced. This meant that 93 % of the basta resistant ku70 transformants were active in RNA silencing.

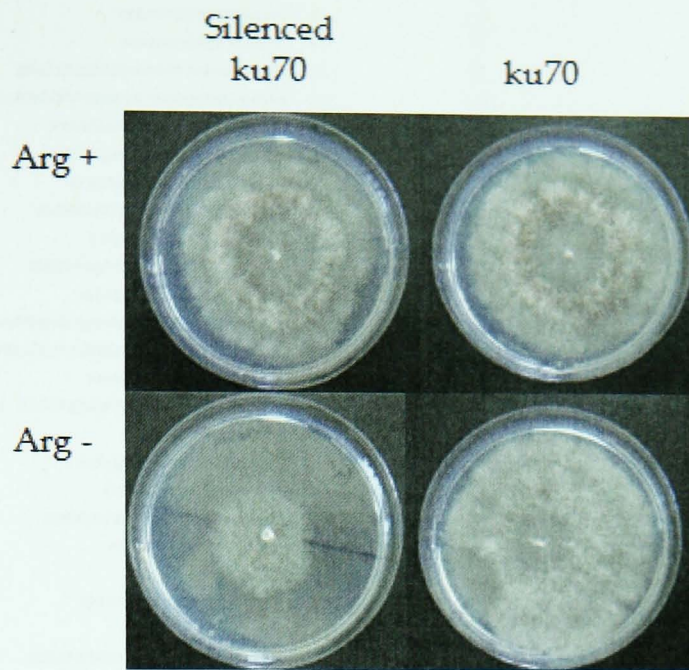


Figure 4.5 Gamborg minimal media assays with and without arginine supplementation for the silenced *B. cinerea* ku70 (Sku70) and parent ku70 showing obvious reduction in growth for the silenced ku70 on media without arginine supplementation.

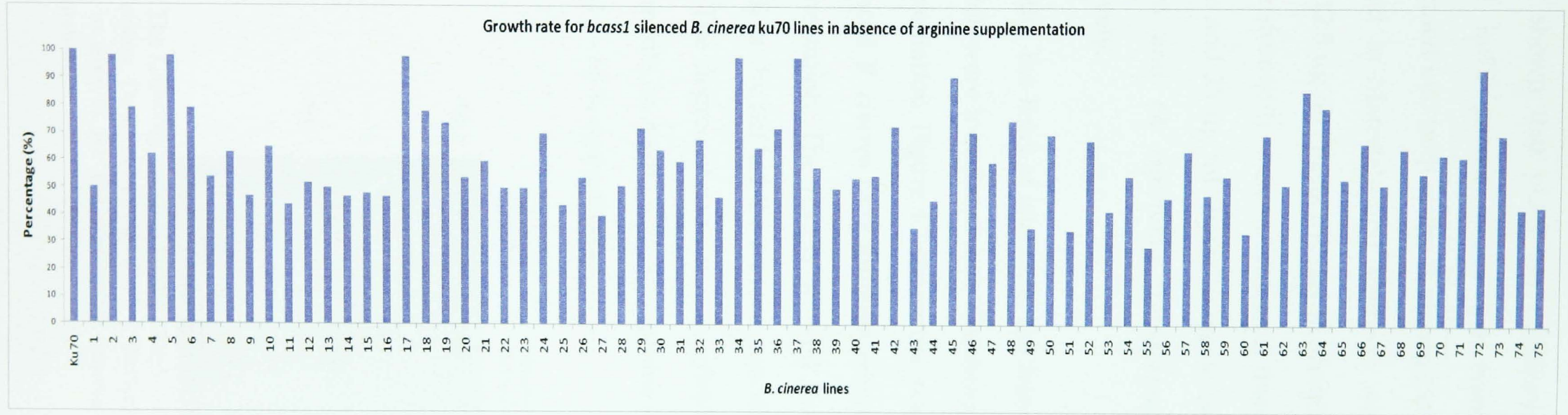


Figure 4.6 The graph shows radial growth (%) of silenced ku70 lines (Sku70) compared to the parent *B. cinerea* ku70 strain when grown on Gamborg media without arginine supplementation.

Having shown that pLOB-bar effectively induced RNA silencing in the original ku70 parent strain, the *bcass1* silencing plasmid was then transformed into both of the *B. cinerea* *Dcr2* mutants. Transformation was as described in Materials and Methods with arginine supplemented media having 12.5 $\mu\text{g ml}^{-1}$ basta overlay to identify possible basta resistant colonies. The transformants were subcultured three times onto media plates having arginine and 25 $\mu\text{g ml}^{-1}$ basta to create basta resistant monokaryons. There were 96 and 64 surviving transformants for BcDcr2-1 and BcDcr2-2 respectively.

To identify the level of silencing, the transformants, and ku70 parent strain as control, were inoculated onto Gamborg plates with and without arginine supplementation. Figure 4.7 shows the visual reduction in growth of some of the silenced *B. cinerea* *Dcr2* mutants when grown in media lacking arginine supplementation. The results showed that all the BcDcr2-1 transformants and 98 % of the BcDcr2-2 transformants showed a silencing effect (Fig 4.8 A and B). The degree of reduction in growth for the BcDcr2-1 and BcDcr2-2 transformants on media without arginine supplementation ranged from 10 – 75 % and 1 - 68 % respectively.

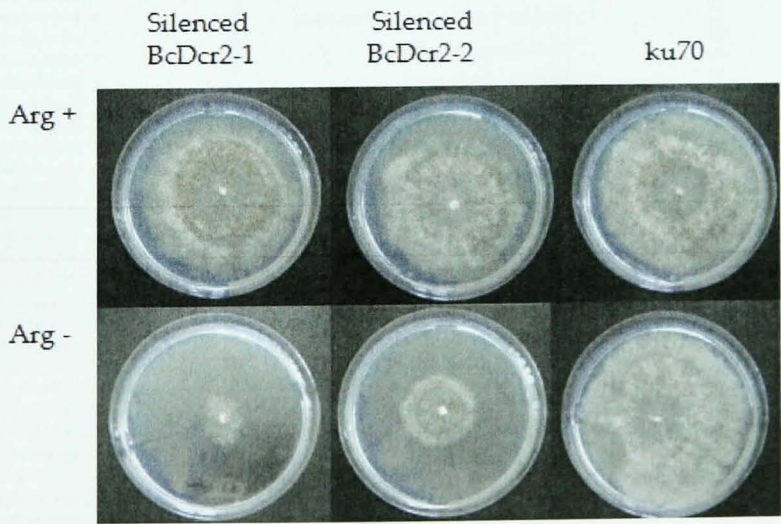


Figure 4.7 The Gamborg minimal media assays with and without arginine for the silenced *bcass1* *B. cinerea* *Dcr2* mutants (BcDcr2-1, BcDcr2-2) and parent ku70 showing obvious reduction in growth for the silenced *B. cinerea* *Dcr2* mutants in absence of arginine supplementation.

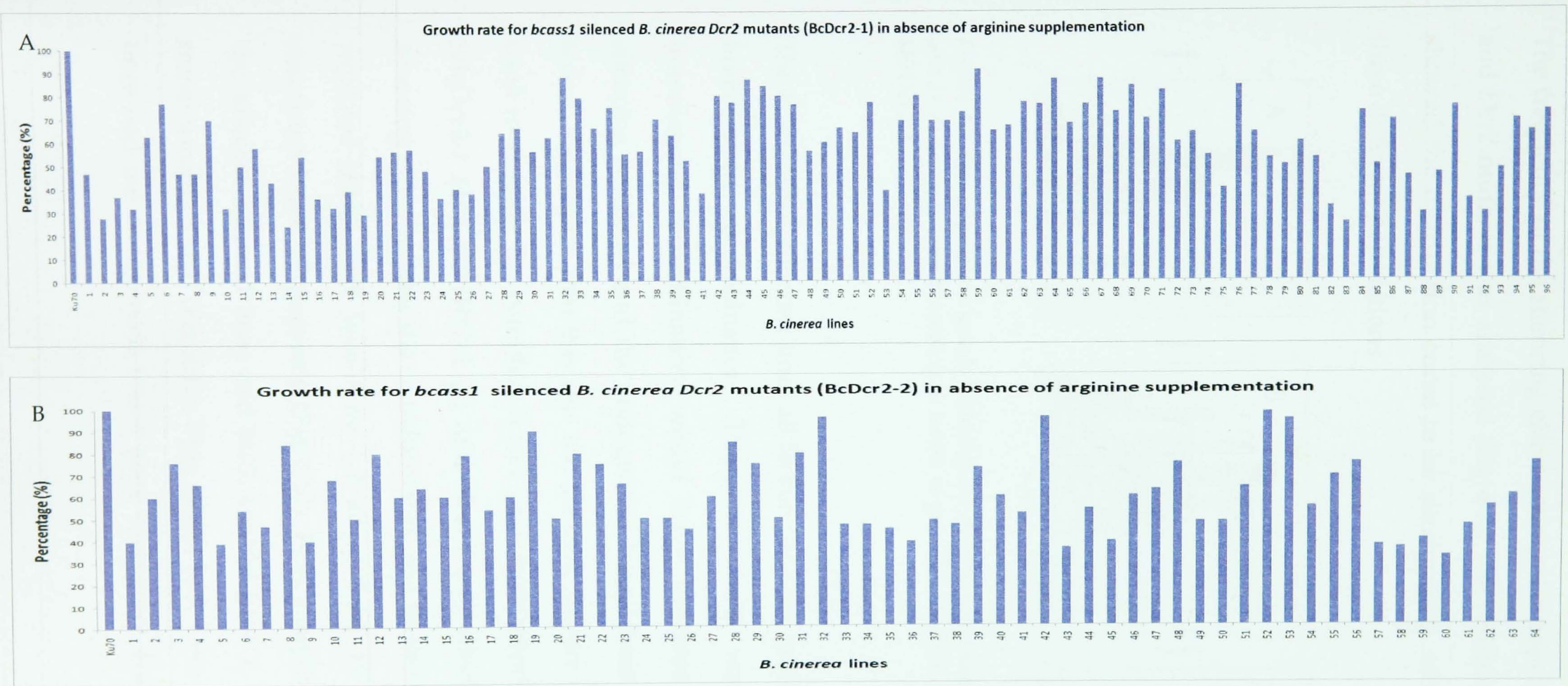


Figure 4.8 The graph shows radial growth (%) on Gamborg minimal media without arginine supplementation for the silenced BcDcr2-1 (A) and BcDcr2-2 (B) lines compared to *B. cinerea* ku70 parent strain.

The frequency of the silencing effect among the population of silenced *ku70* and *Dcr2* mutants was analysed (Fig 4-9). The resulting populations graphs showed the distribution curve to be similar (avg. 60% growth) among the three different populations.

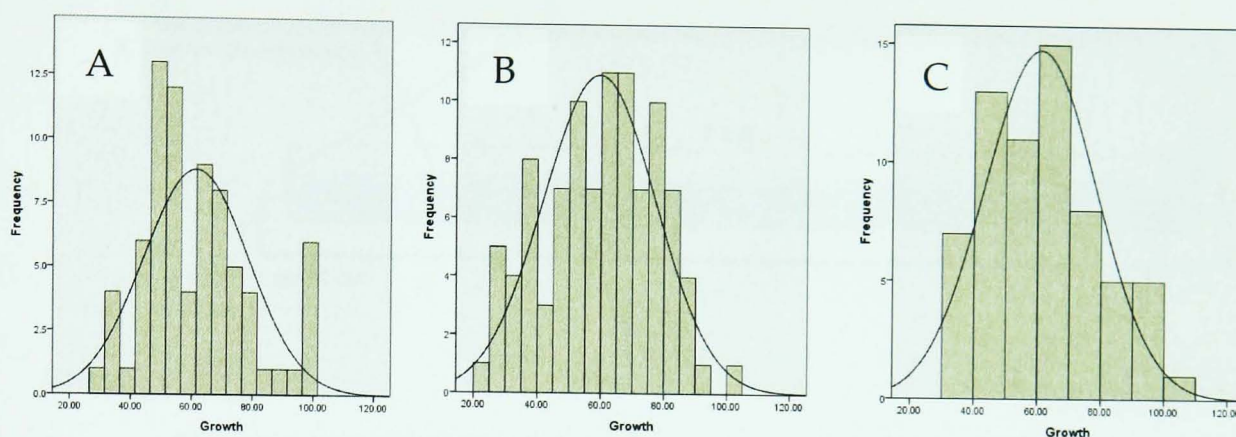


Figure 4.9 The population graph showing the distribution frequency of the silenced *ku70* and *Dcr2* mutants lines according to levels of growth reduction. A: Sku70, B: SBcDcr2-1, C: SBcDcr2-2.

The *bcass1* RNA silenced lines all lacked the *ku70* gene which promotes non-homologous recombination. Therefore, there was a possibility that homologous recombination would have occurred between the *bcass1* silencing construct and the endogenous *bcass1* locus causing disruption of *bcass1*. To confirm that this was not the case, primer set I (Appendix 1) was used to amplify the complete *bcass1* gene from selected RNA silenced lines SBcDcr2-1 (43), SBcDcr2-2 (1) and Sku70 (60) which showed the highest silencing effect among the transformants, and parent *ku70* as a control. The expected band sizes would be 1.2 kb and 6.7 kb for intact *bcass1* and disrupted *bcass1* respectively (Fig 4.10). PCR analysis using primer set I on the selected silenced lines and *ku70* control gave a 1.2 kb band for all the transformants tested (Fig 4.11). This meant that none of the selected silenced lines had the endogenous *bcass1* gene disrupted. Therefore, the phenotypic

effects were most likely due to RNA silencing despite these strains having had disruption in *Dcr2*.

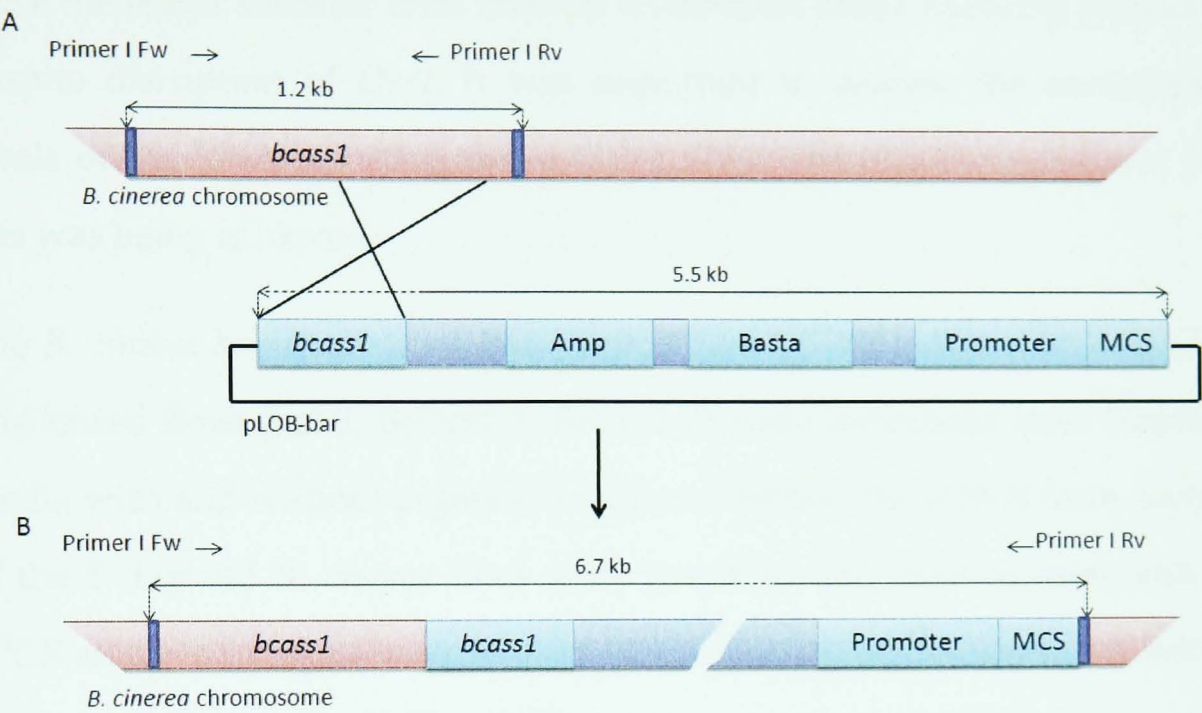


Figure 4.10 To establish whether the endogenous *bcass1* gene was disrupted by transformation with pLOB-bar, primer set I for PCR analysis was designed to amplify a (A) 1.2 kb fragment for a non disrupted and a (B) 6.7 kb fragment for a *bcass1* disrupted transformant. The primers were located in the regions flanking the *bcass1* gene.



Figure 4.11 PCR analyses using primer set I confirm that the *B. cinerea* endogenous *bcass1* gene was not disrupted for the selected *bcass1* RNA silenced lines. L: Hyperladder; 1: SBcDcr2-1, 2: SBcDcr2-2 and 3: ku70, all shows the intact wild-type *bcass1* locus as shown by a product of 1.2 kb.

4.3.5 Real-time PCR analysis on *bcass1* silenced lines

Since the *bcass1* silenced lines showed an obvious *bcass1* silencing phenotype despite disruption of *Dcr2*, it was important to analyse the transcription levels of the RNA silencing genes (*Dcr1*, *Dcr2* and *Argo1*) to discover how this was being achieved.

The *B. cinerea* *bcass1* silenced lines (S-BcDcr2-43, S-BcDcr2-1, S-ku70-60) and unsilenced lines (ku70, BcDcr2-1, BcDcr2-2) were inoculated onto Gamborg media with and without arginine supplementation. The cDNA from mycelia of the 5 day old *B. cinerea* lines were prepared and used as templates for qPCR analysis using primers sets for *Dcr1*, *Dcr2* and *Argo1*, using β -tubulin as the control (Appendix 1). The qPCR reactions were run in triplicates.

The *bcass1* silenced lines all showed a similar small increase in *Dcr1* expression when silencing of *bcass1* was active in the lines, with a further increase when the silenced lines were grown on media lacking arginine supplementation (Fig 4.12). *Dcr2* mutants had the same pattern as the wild type irrespective of silencing state, showing no compensatory increase in *Dcr1* expression despite the loss of *Dcr2*.

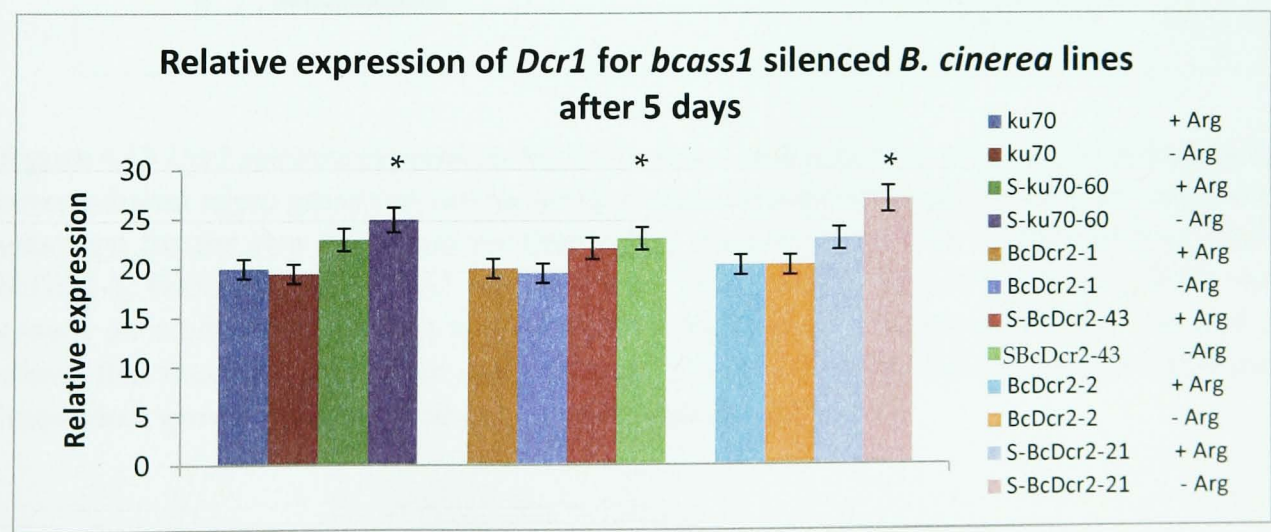


Figure 4.12 The relative expression levels for *Dcr1* for *bcass1* silenced lines showing an increase in expression when *bcass1* was active, and a further increase in expression when

lines were grown on media without arginine supplementation. The unsilenced lines (ku70, BcDcr2-1, BcDcr2-2) and *Bcass1* silenced lines (S-ku70-60, S-BcDcr2-43, S-BcDcr2-21) were grown on media with (+ Arg) and without (- Arg) arginine supplementation. Asterisk (*) shows that there is a significant difference ($p : <0.05$) in *Dcr1* expression between individual lines when grown with and without arginine supplementation.

Dcr2 expression levels showed a similar pattern to *Dcr1* expression, having an increase in the expression during active gene silencing but with a large increase than *Dcr1*, when grown on media without arginine supplementation (Fig 4.13). As expected, there was no expression of *Dcr2* in the disrupted lines. The silenced lines gave similar phenotype patterns on media without arginine supplementation, which meant that the increase in *Dcr2* expression for the silenced ku70 line had no contribution to the phenotype, which brings into question the role of *Dcr2* in RNA silencing.

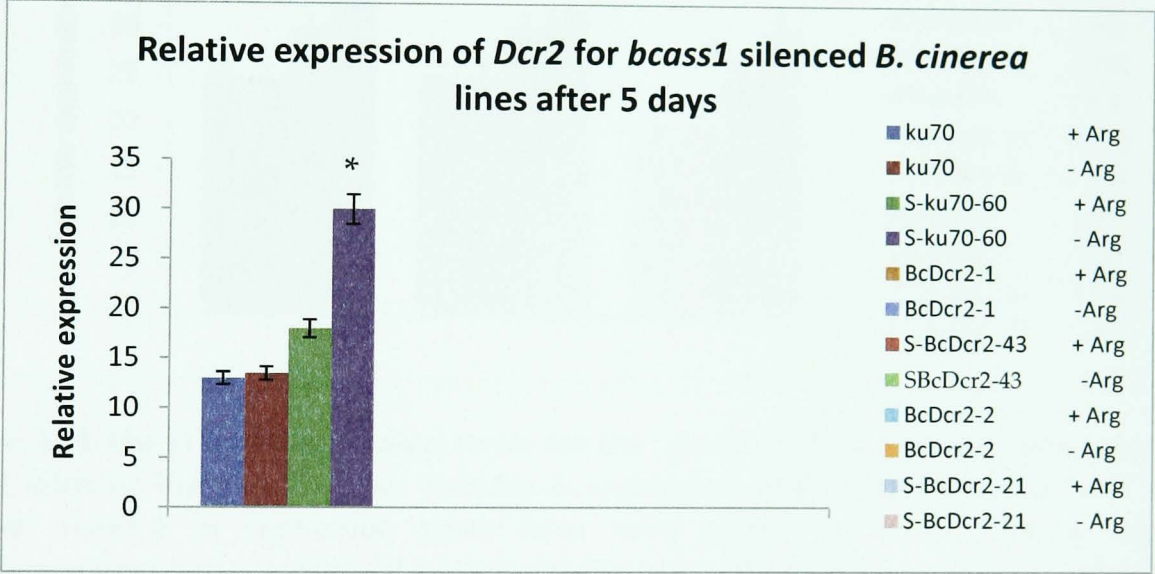


Figure 4.13 *Dcr2* relative expression levels for *bcass1* silenced line, when *bcass1* silencing has been induced when grown on media without arginine supplementation. No *Dcr2* expression was seen for the two *Dcr2* mutants that lacked the *Dcr2* gene. The unsilenced lines (ku70, BcDcr2-1, BcDcr2-2) and *Bcass1* silenced lines (S-ku70-60, S-BcDcr2-43, S-BcDcr2-21) were grown on media with (+ Arg) and without (- Arg) arginine supplementation. Asterisk (*) shows that there is a significant difference ($p : <0.05$) in *Dcr2* expression between individual lines when grown with and without arginine supplementation.

In all the lines analysed, relative expression levels for *Argo1* increased when *bcass1* silencing was induced, with a further increase when the lines were grown on media without arginine supplementation (Fig 4.14). The expression pattern was therefore similar to the increase in *Dcr1* and *Dcr2* expression, illustrating a correlation between the Dicer genes and the *Argo1*.

There was no significant difference in *Dcr1*, *Dcr2* and *Argo1* relative expression levels for the unsilenced lines grown on media with or without arginine supplementation, but the difference for the *bcass1* silenced lines were significant in all cases.



Figure 4.14 The relative expression levels for the putative RNA silencing gene *Argo1* for *bcass1* silenced lines showing an increase in expression when *bcass1* was induced, and a further increase in expression when lines were grown on media lacking arginine supplementation. The unsilenced lines (ku70, BcDcr2-1, BcDcr2-2) and *Bcass1* silenced lines (S-ku70-60, S-BcDcr2-43, S-BcDcr21) were grown on media with (+ Arg) and without (- Arg) arginine supplementation. Asterisk (*) shows that there is a significant difference ($p < 0.05$) in *Argo1* expression between individual lines grown with and without arginine supplementation.

The results from scoring the growth rates for the plate assays and qPCR analysis showed that disruption of the *Dcr2* gene in *B. cinerea* does not inhibit RNA silencing.

4.4 Discussion

4.4.1 RNA silencing of the *bcass1* gene

Basta selection conditions were successfully established in the laboratory, allowing this selectable marker to be used to introduce the pLOB-bar *bcass1* silencing construct into *B. cinerea*. This work used hygromycin, nourseothricin and basta for selection of resistant transformants. Among these selection markers for *B. cinerea*, personal experience has shown that the basta resistance vector is the best. *B. cinerea* basta transformants were easier to identify and produced less false positives among a selected population. Also, since *B. cinerea* is very sensitive to basta, less of it is required in the media for selection.

There has been a lot of interest in the RNA silencing mechanism in *Eukaryotes* including fungi (Schumann *et al.*, 2010). Since it has already been reported that RNA silencing was active in *B. cinerea* (Patel *et al.*, 2008), transforming *B. cinerea* ku70 with the *bcass1* silencing cassette gave the expected *bcass1* silencing effect of reduced mycelial growth on Gamborg media without arginine supplementation.

The RNA silencing effects in this work were broadly similar to those observed by Patel *et al.*, (2010) which used the same *bcass1* silencing cassette. However, the two experiments cannot be directly compared because this work used pLOB-bar to carry both the silencing cassette and basta resistance gene for selection while Patel *et al.*, (2010) used co-transformation with a hygromycin resistance plasmid and a separate pLOB1-MCS for silencing which meant that some transformants would not have acquired the silencing construct.

In most of the filamentous fungi which have been studied, there are two Dicer genes present and disruption of just the Dicer 2 gene was sufficient to inhibit RNA silencing (Abubaker *et al.*, 2010). To determine whether RNA silencing remained active for the *B. cinerea* *Dcr2* mutants, they were transformed with the *bcass1* silencing cassette and the selected transformants were scored for growth on Gamborg media with and without arginine supplementation. Reduction of growth rate in the absence of arginine for the *Dcr2* mutants was unexpected as it was indicative of *bcass1* silencing, suggesting these strains were still able to perform gene silencing.

Observing the *bcass1* RNA silencing effect for the *Dcr2* mutants showed that RNA silencing in *B. cinerea* was not like most of the other fungi studied to date (Kadotani *et al.*, 2004; Segers *et al.*, 2007; Hammond *et al.*, 2008). An *Ascomycete* fungus that showed similar results with this work was *N. crassa*, where it was found that loss of the Dicer 2 gene alone did not inhibit RNA silencing (Catalanotto *et al.*, 2003). Only disruption of both *dcl-1* and *dcl-2* gave a strain unable to silence. The screening of the *N. crassa* mutants impaired in silencing was not able to identify any mutants which lacked a Dicer gene. Since the selection approach used in *N. crassa* relied on mutants showing loss of silencing, the Dicer mutants would not have been identified because loss of both Dicer genes is required before RNA silencing can be inhibited (Catalanotto *et al.*, 2003).

Comparing the frequency with which silencing occurred in the transformants, and strength of the *bcass1* silencing effect, the *ku70* and *Dcr2* populations showed no obvious differences. The majority of transformants showed restricted growth in the absence of arginine supplementation, which correlates closely to silencing of the *bcass1* gene in work by Patel *et al.*, (2010). The frequency of silencing was identical in all three populations, showing

that there was no alteration in silencing, despite deletion of *Dcr2*. The strength of silencing was the same in all populations. Therefore *Dcr2* is not essential either for triggering gene silencing or to control strength of silencing, and that *Dcr1* is sufficient for triggering and maintaining *bcass1* silencing in *B. cinerea*.

This also shows that the use of pLOB-bar to induce *bcass1* silencing in *B. cinerea* gave consistent results among the silenced ku70, BcDcr2-1 and BcDcr2-2 populations. Therefore, this makes pLOB-bar a good candidate for work requiring the induction of *bcass1* silencing in *B. cinerea* strains or as a vector for silencing other genes. Also, pLOB-bar produces antisense transcripts that trigger *bcass1* silencing that gives a strong phenotype. This plasmid would remove any need to make a *bcass1* silencing construct that would produce RNA silencing hairpins in *B. cinerea*.

It was already known that *B. cinerea bcass1* disrupted mutants had a far stronger auxotrophy than *bcass1* silenced lines (Patel *et al.*, 2010), however, the possibility that the highly silenced *bcass1* lines were disrupted mutants was still investigated, given that pLOB-bar was transformed in *B. cinerea Dcr2* mutants, which lacks the ku70 gene. PCR analysis showed that the *bcass1* gene was not disrupted for the selected silenced lines. The phenotypic effect observed was caused by the presence of the *bcass1* silencing cassette being expressed in the lines.

4.4.2 Analysis of *B. cinerea* genes involved in RNA silencing

The qPCR results showed an expected increase in the relative expression levels of *Dcr1* for the *bcass1* silenced parent strain, with a surprising

additional increase in expression when the lines were grown on media lacking arginine supplementation. However, the expression levels for the two silenced *Dcr2* mutants were similar to that of the silenced parent strain, having an increase in *Dcr1* expression when *bcass1* silencing was induced and a further increase when grown on media without arginine supplementation. This showed that there was no significant increase in *Dcr1* expression for the two silenced *Dcr2* mutants, to compensate for the loss of *Dcr2*. Since silencing was active for the *Dcr2* mutants, this showing that the *Dcr1* is sufficient for normal RNA silencing in *B. cinerea*.

If *B. cinerea* is like *N. crassa*, which has the two Dicer genes redundant in RNA silencing, this may explain why there is no inhibition of the transcription levels of the putative RNA silencing genes because the RNA silencing mechanism would still be active even with loss of the *Dcr2* gene. Regulation of Dicer expression has not been investigated in other fungi, but has been studied in human cells. This showed an increase in Dicer expression correlated to the decrease in *let-7* (a negative regulator of *Dcr2*) expression induced by *let-7* antisense construct (Tokumaru *et al.*, 2008). Using BLAST search, *let-7* showed no sequence homology to any gene in *B. cinerea*, suggesting that this regulatory system is not conserved. Since *Dcr2* is dispensable in RNA silencing, the role of the proteins encoded by *Dcr2* in *B. cinerea* would be of interest.

Relative transcription levels for *Argo1* for the *bcass1* silenced lines followed that of *Dcr1* and *Dcr2*, having an increase expression when silencing was active and a further increase on media lacking arginine supplementation. This correlation between the Dicer genes, especially *Dcr1*, and *Argo1* was expected since the proteins encoded by these genes have been known to be involved in the RNA silencing mechanism (Ameres *et al.*, 2011). Therefore,

when *bcass1* silencing was active in *B. cinerea* silenced lines, the increase in Dicer transcript levels was mirrored by *Argo1*.

If the relative expression levels for the RNA silencing genes from the parent strains are to be considered the basal level, this work has illustrated that the expression levels for the RNA silencing genes could be initially increased by induction of *bcass1* silencing, followed by a further increase when grown on media without arginine supplementation. This initial increase in expression shows that the RNA silencing machinery in *B. cinerea*-silenced lines responded to the presence of the *bcass1* silencing inducing construct. The further increase in expression levels when grown on media lacking arginine supplementation was possibly caused by the increase in *bcass1* transcripts due to the organism's requirement for arginine, which was matched by the expression levels for RNA silencing genes.

The *bcass1* silenced lines were not subjected to plant assays since it has already been reported that active *bcass1* RNA silencing in *B. cinerea* in *in planta* assays could result in reduced fungal virulence (Patel *et al.*, 2010).

5 Impacts of *Botrytis* virus F on wild type and *Dcr2* mutant lines

5.1 Introduction

The association of mycoviruses with fungi has been reported in many different species (Pearson *et al.*, 2009). The best studied of these is the interaction between the chestnut blight fungus *Chryphonectria parasitica* and the CHV1-EP713 mycovirus which conferred hypovirulence to the fungus, weakening the fungus, and allowing the chestnut tree to successfully defend itself from infection by the fungus (Segers *et al.*, 2007). This 12.7 kb dsRNA-transmissible mycovirus (Chen and Nuss, 1998) occurs naturally in Europe and has been used to control the spread of *C. parasitica* on chestnut trees in USA (Allemann *et al.*, 1999).

The two commonly used approaches to elucidate the impact mycoviruses have on fungi are to (1) cure the fungus of the mycoviruses or (2) to introduce the mycoviruses into a virus-free fungal host. The effects on the fungus having the mycoviruses removed or added would be observed and analysed using appropriate physiological, pathological and molecular techniques. To cure fungi of dsRNA elements and mycoviruses, several methods have been reported which include conidial and hyphal tip sub-culturing which is effective for some dsRNA elements that are unable to transfer to all the produced conidia and growing hyphal tips, as reported for *Paecilomyces fumosoroseus* (entomopathogenic fungus), *Ceratocystis ulmi* (Dutch elm disease), and *Helibasidium mompa* (root rot fungus) (Azevedo *et al.*, 2000; Pusey and Wilson, 1981; Ikeda *et al.*, 2003). The use of elevated temperatures above that for normal growth of the fungus has sometimes been found to cure fungi of mycoviruses. In *Rhizoctonia solani* (soybean blight

fungus), the fungus lost the dsRNA elements after being incubated at 35 °C for 1 week (Kousik *et al.*, 1994). Exposure to various antibiotics including cycloheximide has also been reported to cure protein mycoviruses from fungi. In *Eukaryotes*, cycloheximide inhibits RNA synthesis by blocking the translational elongation step (Paoletti *et al.*, 2010). Although the mechanism hindering viral replication has not been identified (Schneider-Poetsch *et al.*, 2010), there have been several fungal species cured of mycoviruses using cycloheximide including *C. parasitica*, *S. homeocarpa*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Chalara elegans* (Zhou and Boland, 1997; Fulbright, 1984; Bottacin *et al.*, 1994; Dalzoto *et al.*, 2006; Melzer and Bidochaka, 1998). These cured fungi were sub-cultured for 14 days on growth media containing cycloheximide (20 µg ml⁻¹) except for *C. elegans* which had cycloheximide (3 µg ml⁻¹) and emetine (500 µg ml⁻¹).

The transfection of mycoviruses into mycoviral-free fungal strains would allow the effect, if any, of the mycovirus on the host to be studied. The possible ways for transfecting filamentous fungi includes transfection using partially purified mycoviruses, protoplast fusion and anastomosis. Partially purified mycoviruses are prepared and mixed with protoplasts in the presence of PEG to introduce the mycovirus into the fungus (Howitt *et al.*, 1995). Protoplast fusion allows the mycovirus from a mycovirus infected protoplast to infect a mycovirus-free protoplast, but requires a marker to select strains of the desired genetic background. For anastomosis, the growing hyphae of mycovirus infected strain and mycovirus-free strains fuse and share cytoplasmic material which would include the mycoviruses (Kanematsu *et al.*, 2010), but again requires a selectable host marker.

The mycovirus infected *B. cinerea* RH106-10 strain used in this study was reported to have two flexuous-rod shaped mycoviruses BVF and BVX. These

mycoviruses have been fully sequenced showing BVF and BVX to have ssRNA genomes of 6827 and 6966 nt (Howitt *et al.*, 2001; Howitt *et al.*, 2006). These mycoviruses were selected because they have monopartite genomes making manipulation more amenable and their potential as biological control agents have not been investigated.

The dsRNA mycovirus which conferred hypovirulence to *B. cinerea* CCg425 (Castro *et al.*, 2003) was unavailable for this work. It would have been interesting to determine whether this mycovirus could suppress the RNA silencing mechanism and if it has the potential to be a biological control agent for the control of *B. cinerea*.

Eukaryotes have the RNA silencing machinery which inhibits replication of viruses (Segers *et al.*, 2007). To protect against elimination by RNA silencing, some viruses encode proteins that suppress the RNA silencing mechanism. Suppression of the mechanism may include interfering with dsRNA recognition or cleavage by DICER proteins, which would reduce the accumulation of small RNAs (Colbere-Garapin *et al.*, 2005). The Potyvirus and Cucumber mosaic virus encoded HcPro and 2b respectively, which suppressed the mechanism by reducing the production of small RNA, an important component in the RNA silencing process (Voinnet, 2005). The brome mosaic virus replicated in the endoplasmic reticulum, protecting the viral RNA genome from the host's ribonucleases (Schwartz *et al.*, 2002). It would be interesting to see if the *B. cinerea* mycoviruses used in this study have the ability to suppress silencing mechanisms.

5.2 Aims

To date, there has been no report of an RNA mycovirus as a biological control agent for *B. cinerea*. This work looked at the potential of the ssRNA mycovirus BVF as a control for the fungus. Therefore the aims for this study were to:

- Compare healthy and infected *B. cinerea* strains
- investigate mycoviral infection in *B. cinerea Dcr2* mutants, and
- analyse gene expression changes due to mycoviral infection.

5.3 Results

5.3.1 *In planta* assays on mycovirus infected *B. cinerea* strains

The mycovirus infected *B. cinerea* strains isolated from fields in New Zealand RH106-10 (strawberry), RH105-19 (strawberry), REB205-10 (grape) and REB171-3 (grape) (Howitt *et al.*, 1995) were subjected to *in planta* assays to determine their virulence levels compared to the *B. cinerea* B05-10 laboratory strain. The assays were set up as described in Materials and Methods with the B05-10 control inoculated on the left side of the mid-rib, and on the right, the tested strain. The plants selected for leaf assays were French dwarf beans (var. Tender green) and green pepper (var. Sweet rainbow), and for the fruits, tomato, white grapes and strawberries were used (Fig 5.1 A, B). Data from lesion radius measurements after 5 days are shown in figure 5-2 A and B. Statistical analysis (Student *t*- test $p > 0.05$) on the results showed that there was no significant difference in lesion radius between the mycoviral infected *B. cinerea* strains and the wild type strain B05-10, irrespective of the geographical, host origin or viral content.

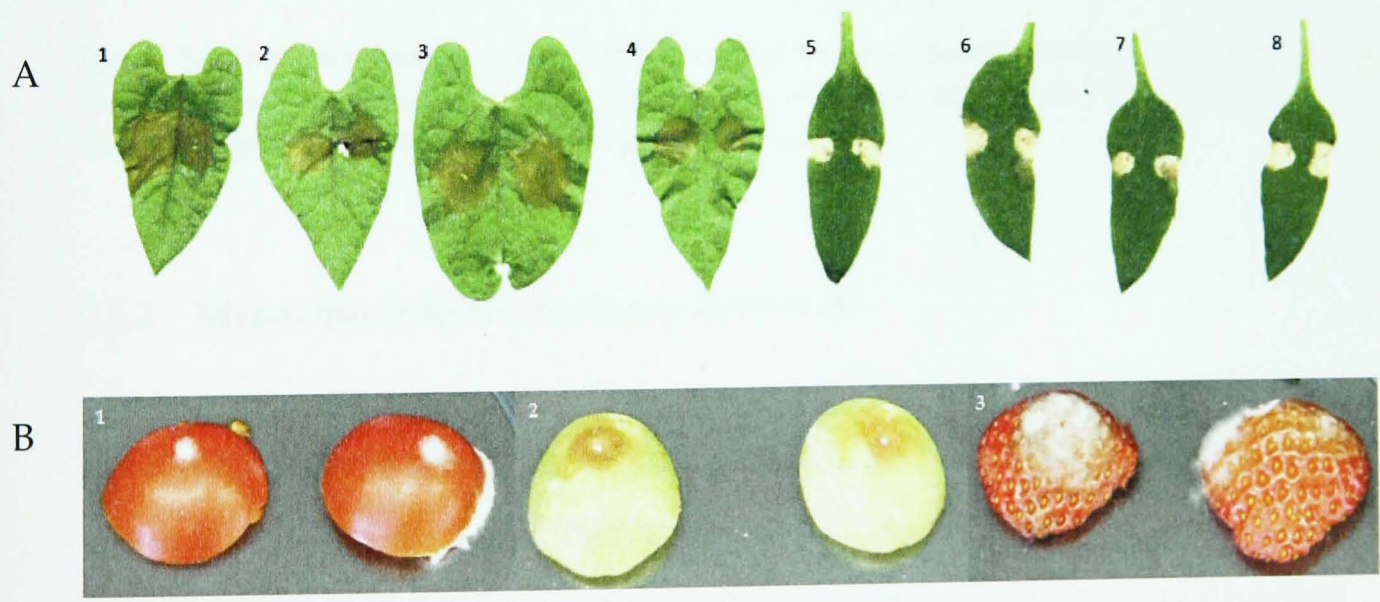


Figure 5.1 Examples of plant leaves and fruit assays to determine level of virulence for the mycoviral infected *B. cinerea* strains compared to *B. cinerea* B05-10. A: French dwarf bean leaves (1 - 4), green pepper leaves (5-8), B: 1- Tomato; 2- white grapes and 3- strawberry. A: Left side B05-10, Right side RH106-10, RH105-19, REB205-10 and REB171-3 respectively. B: Left B05-10 compared to RH106-10.

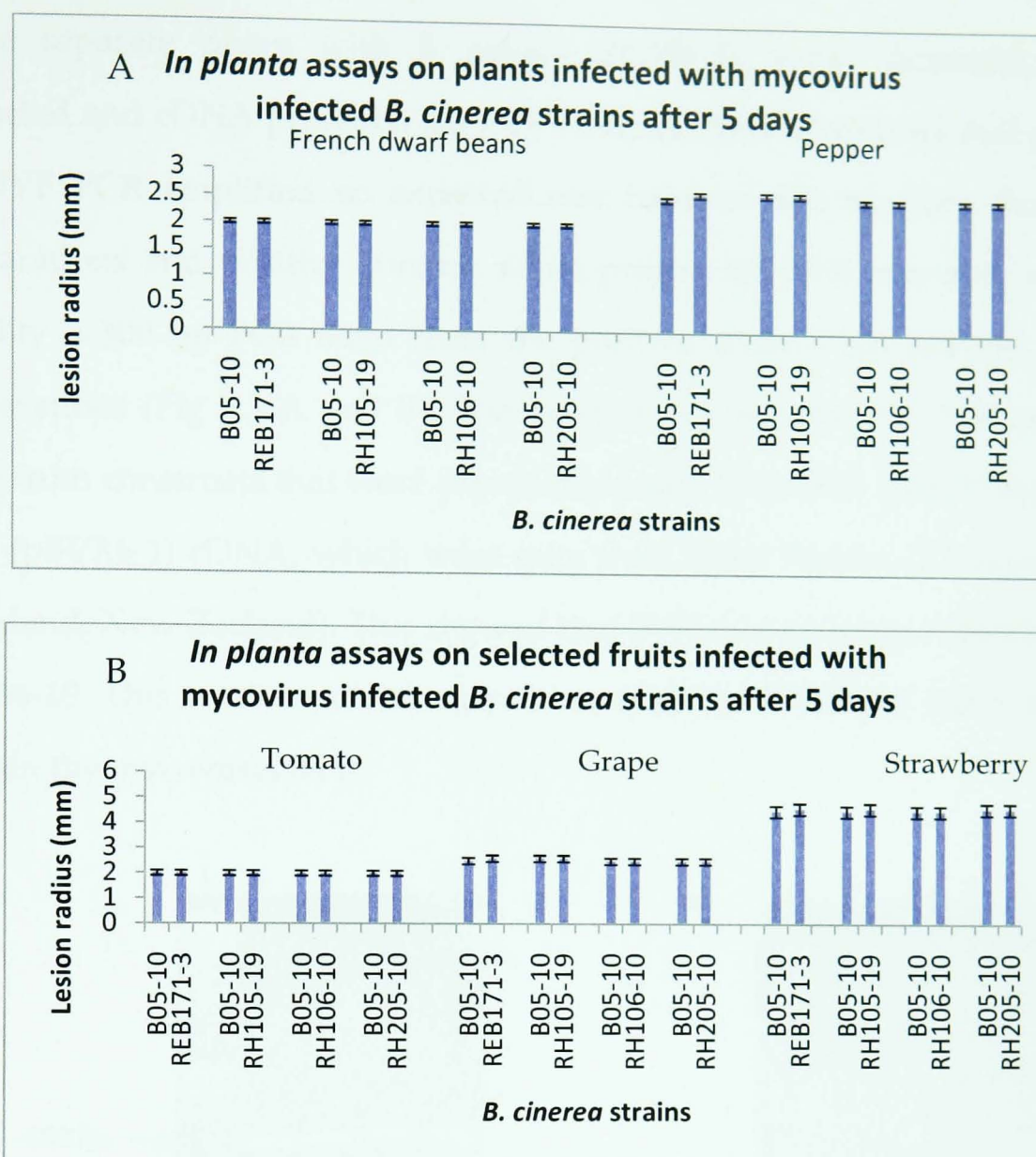


Figure 5.2 *In planta* assays on selected leaves and fruits to determine the level of virulence for the mycoviral infected strains compared to *B. cinerea* B05-10. A: French dwarf bean leaves, pepper leaves; B: tomatoes, grapes and strawberry.

5.3.2 Mycovirus infected *B. cinerea* RH106-10

Two fully sequenced ssRNA mycoviruses *Botrytis* virus F and X (BVF, BVX) were identified in *B. cinerea* RH106.10 (Howitt *et al.*, 1995). Before experimentation, primer sets (Appendix 1) for BVF and BVX were designed from sequences available on the NCBI database (accession numbers BVF: AF238884, BVX: AY055762) to determine whether the mycoviruses remained present in the strain.

Three separate plates with *B. cinerea* RH106-10 were harvested, RNA extracted and cDNA prepared for PCR analysis. Results showed that primer set BVF PCR amplified an expected size band of 400 bp from the three preparations and positive control, while primer set BVX was only able to amplify a 300 bp PCR band from the positive control but not the cDNA preparations (Fig 5.3 A and B). The positive controls for the PCR analysis were from constructs that were previously made from BVF (pTOPO6-1) and BVX (pBVX6-1) cDNA, which were gifts from Mike Pearson (University of Auckland, New Zealand). This showed that BVX was no longer detectable in RH106-10. This work would therefore consider the RH106-10 strain to only contain the mycovirus BVF.

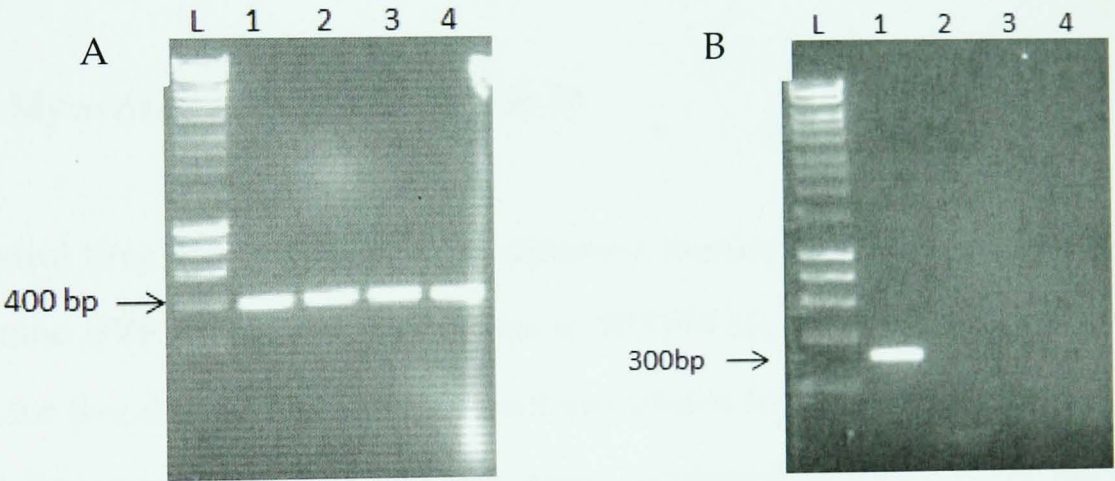


Figure 5.3 The electrophoresis gels showing that BVF (A) but not BVX (B) was detectable by PCR analysis using primer sets for BVF and BVX in RH106-10 cDNA preparations. A: L – Hyperladder, 1- BVF construct (Control), 2-4 – RH106-10 cDNA preparations; B: L – Hyperladder, 1- BVX construct (Control), 2-4 – RH106-10 cDNA preparations.

The *B. cinerea* B05-10 lab strain was also analysed by PCR to determine whether it contained BVF and BVX. Results showed that BVF and BVX primer sets amplified expected size bands from pTOPO6-1 (400 bp) and pBVX6-1 (300 bp), while no virus-derived PCR products were ever produced from *B. cinerea* B05-10 cDNA (Fig 5.4). This shows that *B. cinerea* B05-10 can

be used as a virus-free recipient in experiments with BVF and BVX infected *B. cinerea*.

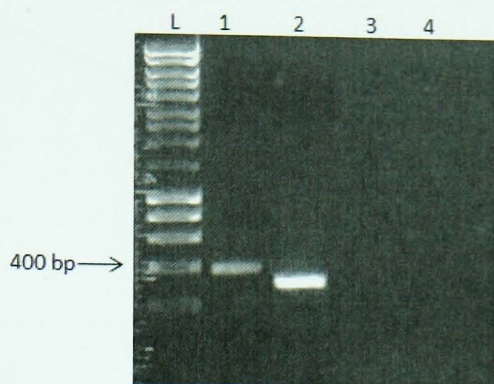


Figure 5.4 PCR analysis to determine whether BVF and BVX were present in *B. cinerea* B05-10. BVF primer set PCR amplified the expected 400 bp band from pTOPO6-1 (Lane 1), but not from B05-10 cDNA (lane 3). BVX primer set PCR amplified the expected 300 bp from pBVX6-1 (Lane 2), while no product was produced using B05-10 cDNA as a template. L: Hyperladder.

5.3.3 Mycoviral titre levels in RH106-10

Mycoviral titre levels may vary in different tissues or at different times. To determine BVF mycoviral titre levels in RH106-10, conidia grown on MEA plates for five days were collected and inoculated into conical flasks having 1 % ME liquid media. The cultures were incubated at 25°C. Cultures were harvested at 0 h followed by 12 h intervals and cDNA prepared for qPCR analysis. Using BVF and β tubulin as the control (Appendix 1) primer sets, the titre levels for BVF was determined by qPCR over the course of 72 h at 12 h intervals. The results showed that there was a reduction in relative mycoviral titre levels after 12 h, with the lowest level at 48 h, followed by a steady increase (Fig 5.5).

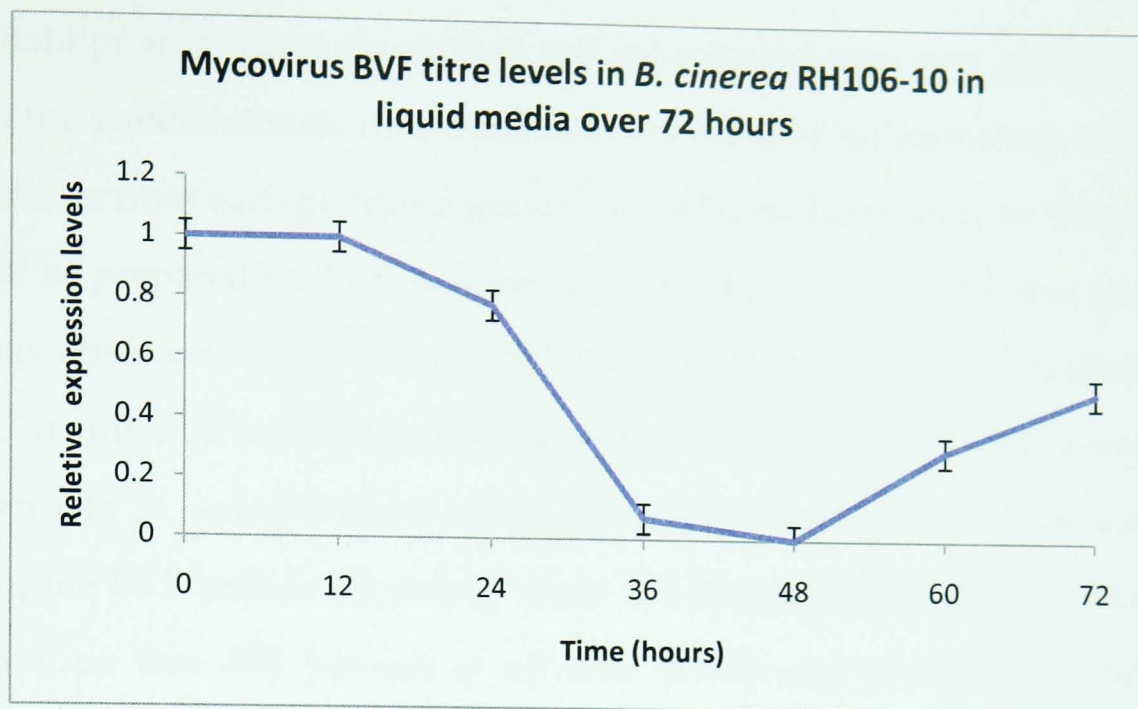


Figure 5.5 Relative BVF mycoviral RNA levels in *B. cinerea* RH106-10 in liquid media in triplicates over 72 h at 12 h intervals measured by qPCR.

5.3.4 Curing mycovirus infected *B. cinerea* RH106-10

Although the bioassays for *B. cinerea* RH106-10 and B05-10 were similar (5.3.1), these are genetically different strains, from different hosts and different locations. It is possible that the presence of the BVF in RH106-10 may be restricting the fungus from increased virulence. To determine whether this is the case, RH106-10 was grown on media containing the antibiotic cycloheximide in an attempt to cure the fungus of BVF.

The method of Zhou and Boland (1997) was used since it was reported to be successful in curing *Sclerotinia homoeocarpa*, a close relative of *B. cinerea*, of mycoviral particles. This method was based on repeated subcultures in the presence of cycloheximide. Malt extract media with cycloheximide concentrations of 0, 0.5, 2.5, 5, 12.5, 25, 35, 50, 100 $\mu\text{g ml}^{-1}$ were point inoculated with mycoviral *B. cinerea* RH106-10. The experiment was carried out at two incubation temperatures (25°C and 30°C). After 4 days growth,

hyphal tips were carefully excised and subcultured onto new growth plates with the same antibiotic concentrations. On the third subculturing, a sample was taken from each plate and grown on antibiotic-free plates, so that cDNA could be prepared for PCR analysis to determine whether BVF was present. It was observed that on all plates having cycloheximide and incubated at 30°C, no mycelia were seen growing. For plates incubated at 25°C, there was no growth at concentrations above 25 µg ml⁻¹. Results from PCR analysis using the BVF primer set clearly show 400 bp products indicating that the mycovirus was still present at all time points and at all cycloheximide concentrations which allowed growth (Fig 5.6). This shows that BVF was still able to replicate and be transferred through hyphal subculturing when exposed to cycloheximide concentrations up to 25 µg ml⁻¹ at 25°C.

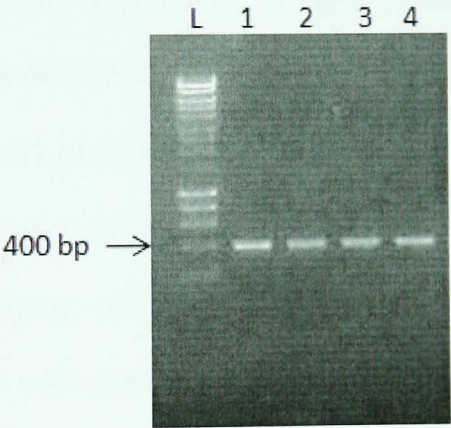


Figure 5.6 Example of an electrophoresis gel showing PCR amplification of a 400 bp band from RH106-10 cDNA using BVF primer set from mycelia growing on media with 25 µg ml⁻¹ cycloheximide. Presence of the band showed that the BVF mycovirus was present in *B. cinerea* RH106-10 after several subcultures on growth plates which had the antibiotic cycloheximide up to concentrations of 25 µg ml⁻¹ at 25 °C. L: Hyperladder, 1:BVF control, 2-4: RH106-10 cDNA preparations.

5.3.5 Transfecting *B. cinerea* with mycovirus BVF

Botrytis cinerea RH106-10 may have reduced virulence, making it now have virulence similar to that of the laboratory strain *B. cinerea* B05-10 due to the presence of BVF. To determine whether BVF confers hypovirulent traits to *B. cinerea*, BVF was introduced to mycovirus-free *B. cinerea* lines. The mycoviruses were partially purified from *B. cinerea* RH106-10 following the methodology of Howitt *et al* (2006) with alterations as described in Materials and Methods. A sample of the partially purified mycoviral suspension was viewed under a transmission electron microscope and 720 nm flexuousrod-shaped mycoviruses were observed; typical for mycovirus BVF (data not shown). If BVX was present, they could not have been distinguished from BVF since both are flexuousrod-shaped mycoviruses. The partially purified mycoviruses were mixed with *B. cinerea* B05-10 protoplasts followed by PEG fusion and allowed to regenerate in SH agar media. Twelve regenerated lines were randomly selected and prepared for PCR analysis using BVF primers to detect the presence of the mycovirus. After several transfection attempts and random selection of emerging colonies, no colony was shown to be infected with BVF by PCR analysis using BVF primers (Fig 5.7).

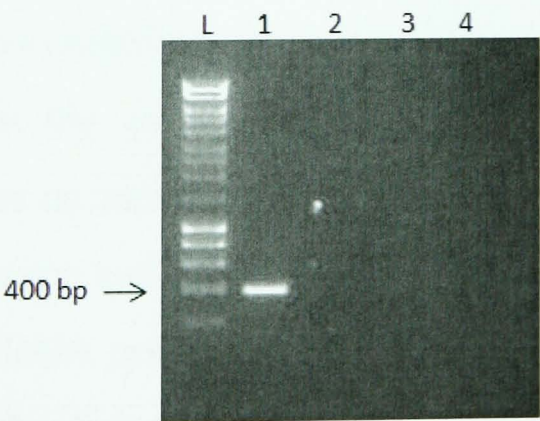


Figure 5.7 PCR analysis using a BVF primer set did not detect any BVF in transfectant B05-10 *B. cinerea* lines. L: Hyperladder, 1: BVF control construct, 2-4 *B. cinerea* lines cDNA preparations of transfectant B05-10.

Having failed to transfect protoplasts with pure mycoviruses, the next transfection approaches used were protoplast fusion and anastomosis as described in Materials and Methods. The recipient host was *B. cinerea* BH-1, a hygromycin resistant transformant of B05-10 made using pOlihygtrpc. This was chosen so that growth on media containing hygromycin would only allow this genotype to proliferate while selecting against any nuclei remaining from the mycoviral donor. BH-1 was transfected with BVF by protoplast fusion which allows the mycovirus in *B. cinerea* RH106-10 protoplasts to enter into the recipient BH-1 protoplasts when the protoplasts fuse together. The mycoviruses are able to transfer among protoplasts because the cell wall barrier has been removed. In the presence of PEG and CaCl_2 , BH-1 protoplasts (6×10^3) and RH106-10 protoplasts (6×10^6) were mixed into molten SH-regeneration media, giving a virus free: infected ratio of 1 : 1000 to maximise likelihood of fusion to allow viral transmission. An overlay of SH containing $50 \mu\text{g ml}^{-1}$ of hygromycin was used and about 50 emerging colonies were subcultured three times on MEA having $100 \mu\text{g ml}^{-1}$ hygromycin with the final subculturing on growth media without antibiotic.

For transfection by anastomosis, the *B. cinerea* RH106-10 donor and BH-1 recipient lines were inoculated on opposing sides of the media plates. A small plug taken from the area where the opposing hyphae meet was subcultured three times on media containing $100 \mu\text{g ml}^{-1}$ hygromycin. Six lines from protoplast fusion and six lines from anastomosis were selected for further analysis, and cDNA prepared for PCR analysis for the presence of BVF. Three of the twelve lines were positive for BVF; one from protoplast fusion and two from anastomosis (Fig 5.8).

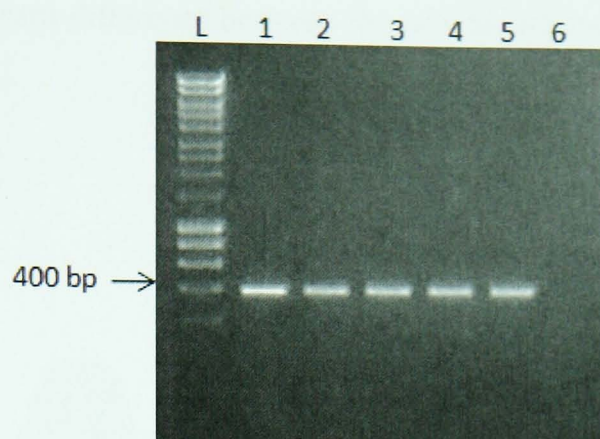


Figure 5-8 PCR analysis using BVF primer sets on cDNA prepared from transfected *B. cinerea* lines. L: Hyperladder, 1: Positive control, 2: RH106-10 donor, 3-5: transfected recipient lines, 6: non-transfected BH-1.

One transfectant (V-BH1) from anastomosis was used in bioassays to determine growth rate and level of virulence compared to *B. cinerea* BH-1. For growth rate analysis, *B. cinerea* strains were inoculated onto MEA plates and radial mycelial growth were recorded after 4 days. Lesion diameter on French dwarf beans inoculated with the *B. cinerea* lines was recorded after 5 days. Statistical analysis ($p < 0.05$) on the data showed that there was no significant difference in growth rate (Fig 5.9) or the level of virulence for the BVF infected line compared to the uninfected strain BH-1 recipient (Fig 5.10 A and B).

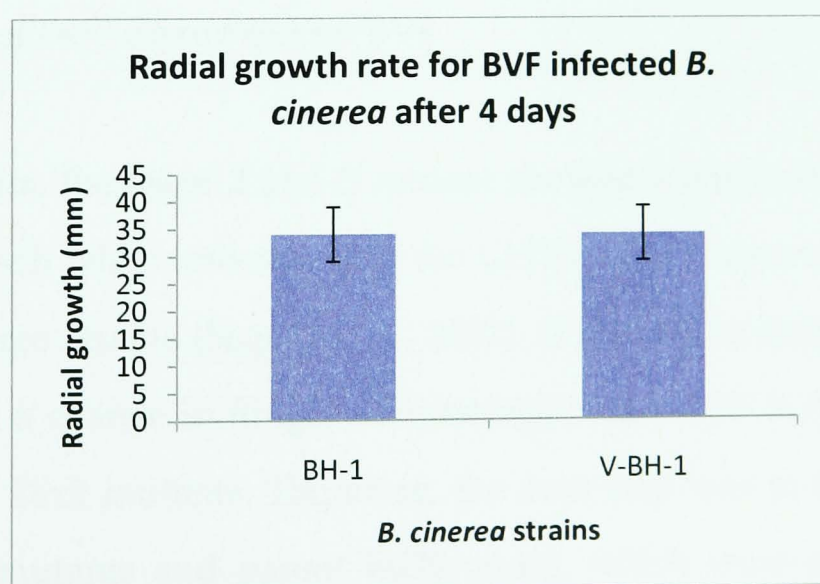


Figure 5.9 Radial growth of BVF infected (V-BH-1) and uninfected (BH-1) *B. cinerea* grown on MEA measured after 5 days. Statistical analysis (Student t-test $p < 0.05$) results showed

that there was no significant difference between the uninfected and BVF infected *B. cinerea* strains.

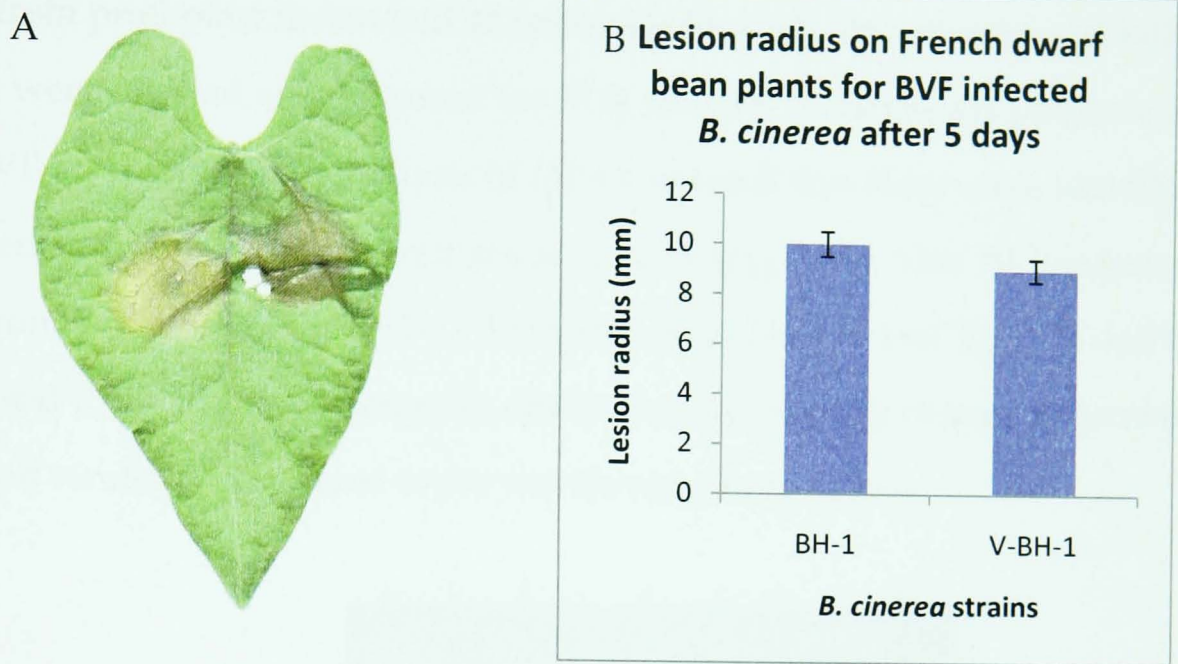


Figure 5.10 French dwarf bean showing fungal lesions after 5 days. A: Example of a *B. cinerea* lesions on leaf inoculated with uninfected (left of midrib) and BVF infected (right of midrib) *B. cinerea* BH-1. B: Lesion diameter for the uninfected and BVF infected *B. cinerea* lines on the plant leaves.

5.3.6 Effect of *Dcr2* disruption on BVF

For *C. parasitica*, the Dicer 2 (*dcl-2*) mutant showed a noticeable reduction in mycelial growth when infected with the CHV1-EP713 mycovirus compared to the virus free strains (Segers *et al.*, 2007). If this is the same in *B. cinerea*, there may be a change in fungal morphology when BVF is introduced into the *B. cinerea* *Dcr2* mutants. Therefore, the next step was to transfect the *B. cinerea* *Dcr2* mutants and parent ku70 strain, which were all hygromycin resistant, with mycovirus BVF by protoplast fusion and anastomosis.

After transfection, about 32 hygromycin resistant colonies from protoplast fusion and 30 plugs from anastomosis were subcultured three times on MEA plates containing $100 \mu\text{g ml}^{-1}$ hygromycin to remove heterokaryon contamination from the mycovirus donor strain. Six hygromycin resistant lines from protoplast fusion and anastomosis for each *Dcr2* mutant and ku70 strain were selected and prepared for PCR analysis to assess the presence of the BVF mycovirus. PCR analysis of cDNA showed that there were two BVF infected lines for each *Dcr2* mutant and ku70 (Fig 5-11). One BVF-infected line from BcDcr2-1 (V-BcDcr2-1), BcDcr2-2 (V-BcDcr2-2) and ku70 (V-ku70) was used for *in planta* bioassays to determine any possible change in growth rate and virulence compared to the uninfected parent strains.

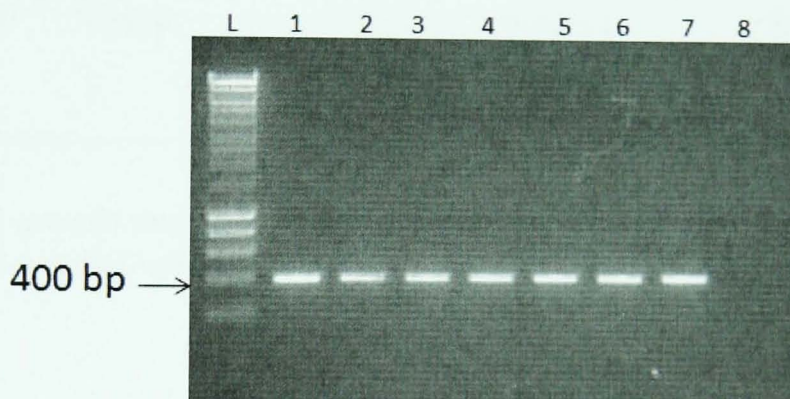


Figure 5.11 Confirmation of BVF in transfected *B. cinerea* *Dcr2* mutants and ku70. L: Hyperladder, 1: Positive control, 2-3: V-BcDcr2-1, 4-5: V-BcDcr2-2, 6-7: V-ku70, 8: virus-free recipient-ku70.

To assess whether BVF impacts on growth in the *Dcr2* mutants, MEA plates were inoculated with the BVF infected lines (V-ku70, V-BcDcr2-1, V-BcDcr2-2) in triplicates. After 4 days growth, radial growth rates were recorded and analysed (Fig 5.12). Results showed that there was no significant ($p < 0.05$) difference in radial growth rate between any of the lines irrespective of mycoviral infection.

The fungal virulence bioassays were carried out on French dwarf bean plants. BVF infected *Dcr2* mutants and the parent ku70 (control) were

inoculated onto the leaves as described in Materials and Methods. The lesion diameter was recorded after 5 days growth (Fig 5.13 A and B). The results showed that there was no significant difference in the level of virulence for the BVF infected *Dcr2* mutants and infected ku70 compared to the uninfected controls.

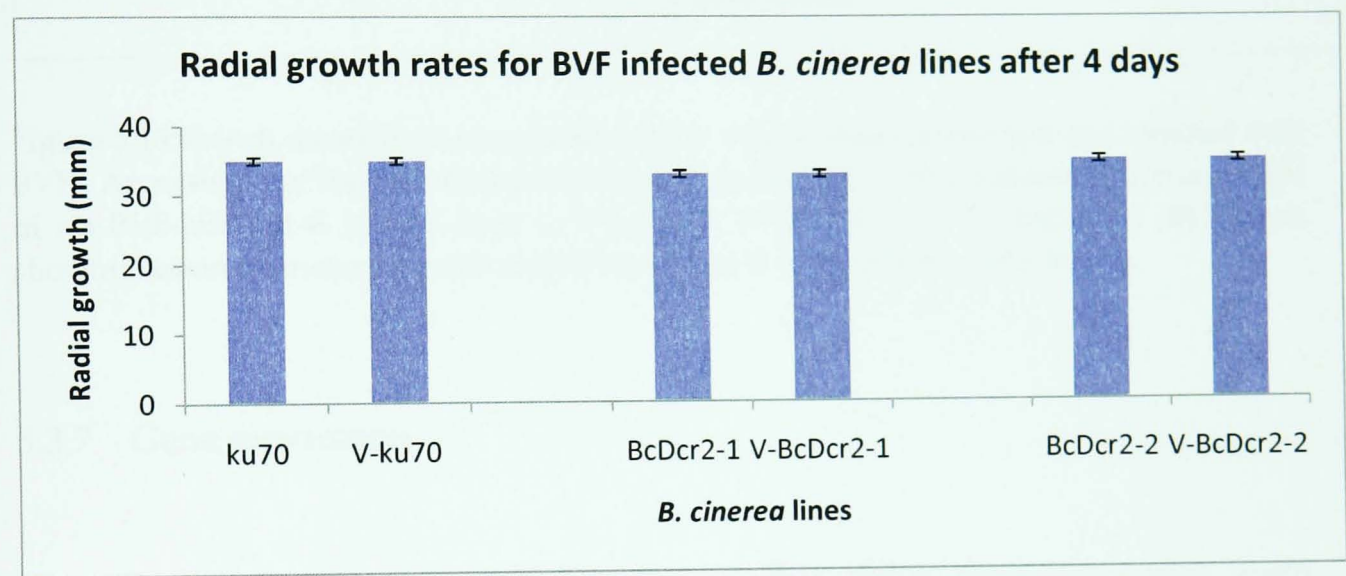


Figure 5.12 Radial growth rate for uninfected (ku70, BcDcr2-1, BcDcr2-2) and BVF infected (V-ku70, V-BcDcr2-1, V-BcDcr2-2) *B. cinerea* lines grown on MEA growth media for 4 days.



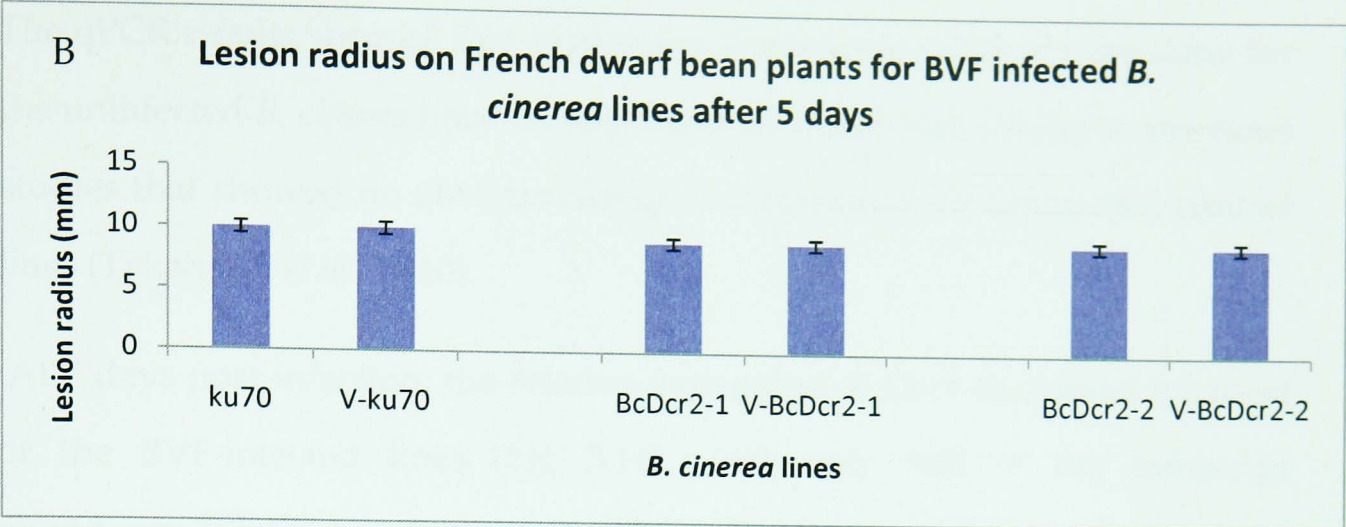


Figure 5.13 French dwarf bean inoculated with *B. cinerea* lines (uninfected and infected with BVF). An example of leaves infected with *B. cinerea*; (A) Left of rib uninfected ku70 and right of rib BVF-infected *B. cinerea* lines 1: V-ku70, 2: V- BcDcr2-1, 3: V- BcDcr2-2. (B). Graph showing lesion diameter for ku70 and BVF-infected *B. cinerea* lines on the leaves.

5.3.7 Gene expression

The relative expression levels for the selected RNA silencing genes were determined for the BVF infected lines compared to the uninfected lines. This was done to determine whether mycovirus BVF had any impact on the expression of the RNA silencing genes. The *B. cinerea* lines were grown and cDNA prepared as described in materials and methods for each BVF-infected line (V-BH-1, V-ku70, V-BcDcr2-1, V-BcDcr2-2) and uninfected lines (B05-10, ku70, BcDcr2-1, BcDcr2-2). For each line, qPCR analysis was performed using primer sets for *Dcr1*, *Dcr2*, *Argo1* and β -*tubulin* with three technical replicates to confirm accuracy of results. Two sets of experiments were carried out, first set used transfected *B. cinerea* lines harvested after 7 days growth on MEA media and the other set, which was subcultured every 7 days, was harvested at 28 days post- transfection to compare consistency of results over time.

The qPCR results showed that expression levels were relatively the same for the uninfected *B. cinerea* lines on day 7 and 28 which was similar to previous studies that showed no obvious change in expression for uninfected control lines (Takahashi *et al.*, 2010).

At 7 days post-infection, the relative expression of *Dcr1* decreased for most of the BVF-infected lines (Fig 5.14), with only half of the transcript abundance for *Dcr1* in both of the BVF-infected *Dcr2* lines. The two *Dcr2* mutants, which lacked the *ku70* gene, showed no increase in expression levels, unlike the uninfected lines, the significance of this is unknown. This shows that the presence of BVF could be having an effect on expression of *Dcr1*.

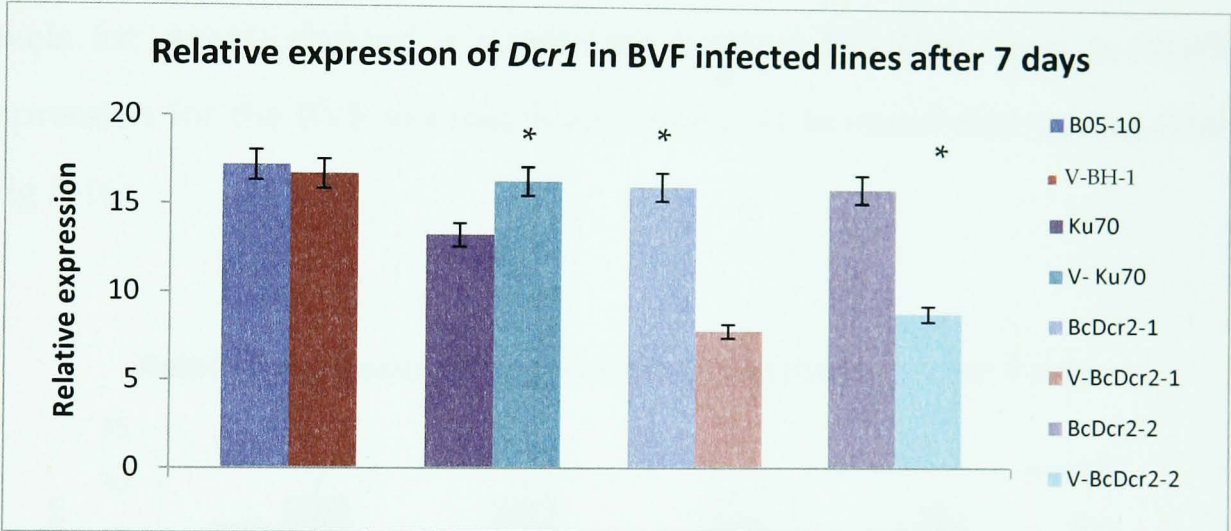


Figure 5.14 The relative expression levels for *Dcr1* at 7 days for BVF infected (V-BcDcr2-1, V-BcDcr2-2, V-BH-1 and V-ku70) and uninfected (B05-10, ku70, BcDcr2-1, BcDcr2-2) *B. cinerea* lines. Asterisk (*) shows that there is a significant difference ($p : <0.05$) in *Dcr1* expression between individual lines when grown with and without mycovirus infection.

For *Dcr2*, there was a decrease in expression when strains were mycovirus infected. The decrease was stronger than that of *Dcr1* with V-BH-1 having half of the remaining transcript, while V-ku70 had only one quarter of the remaining transcript compared to the uninfected pairs of lines (Fig 5.15).

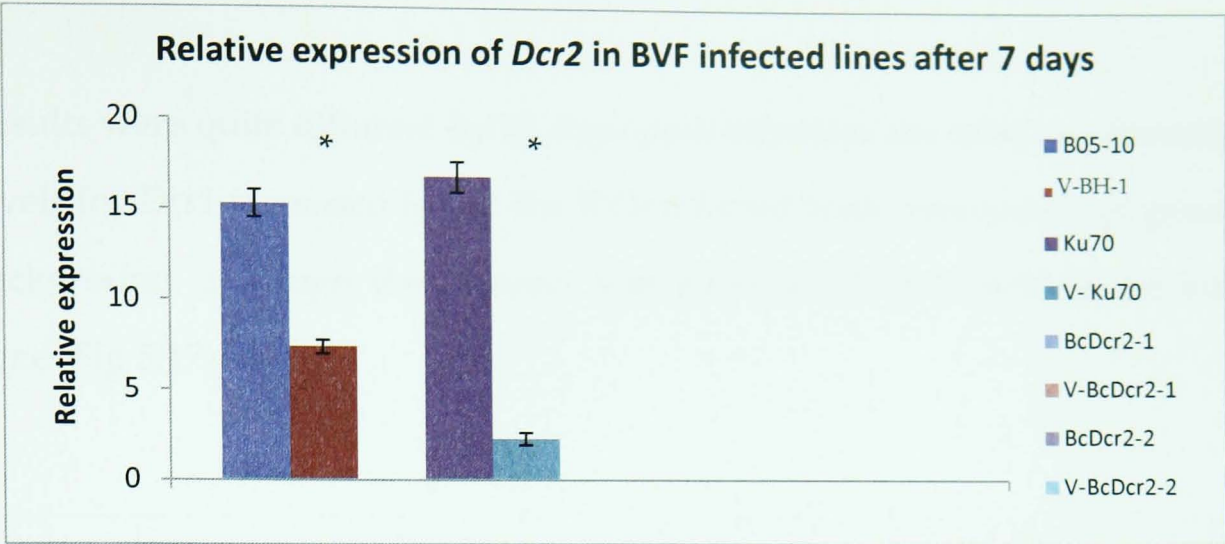


Figure 5.15 *Dcr2* expression levels at 7 days for BVF infected (V-BcDcr2-1, V-BcDcr2-2, V-BH-1 and V-ku70) and uninfected (B05-10, ku70, BcDcr2-1, BcDcr2-2) *B. cinerea* lines. Asterisk (*) shows that there is a significant difference ($p : <0.05$) in *Dcr2* expression between individual lines when grown with and without mycovirus infection.

In contrast to the effects seen on BcDcr2-1 and BcDcr2-2, the expression levels for *Argo1*, showed a significant increase (Student t-test $p: <0.05$) in expression for the BVF-infected lines compared to uninfected parent strains (Fig 5-16).

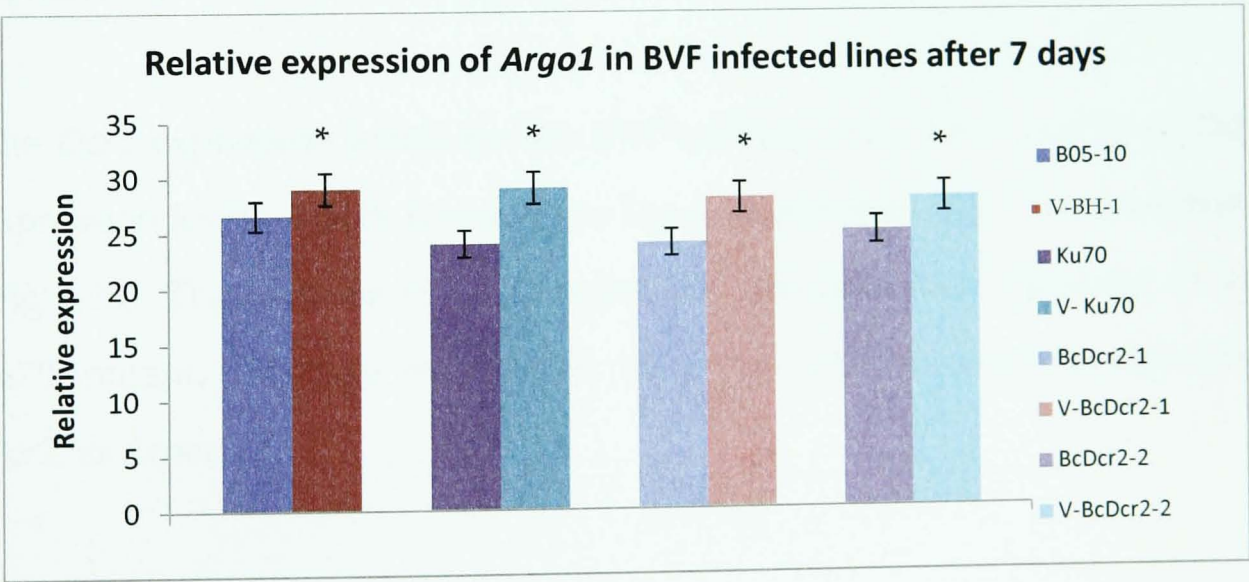


Figure 5.16 The graph shows relative expression levels for *Argo1* at 7 days for BVF infected (V-BcDcr2-1, V-BcDcr2-2, V-BH-1 and V-ku70) and uninfected (B05-10, ku70, BcDcr2-1, BcDcr2-2) *B. cinerea* lines. Asterisk (*) shows that there is a significant difference ($p : <0.05$) in *Argo1* expression between individual lines when grown with and without mycovirus infection.

Results were quite different by 28 days post-infection, the relative expression levels for *Dcr1* increased for all the BVF-infected lines irrespective of genetic background, although the increase was stronger in lines lacking the *ku70* gene (Fig 5.17).

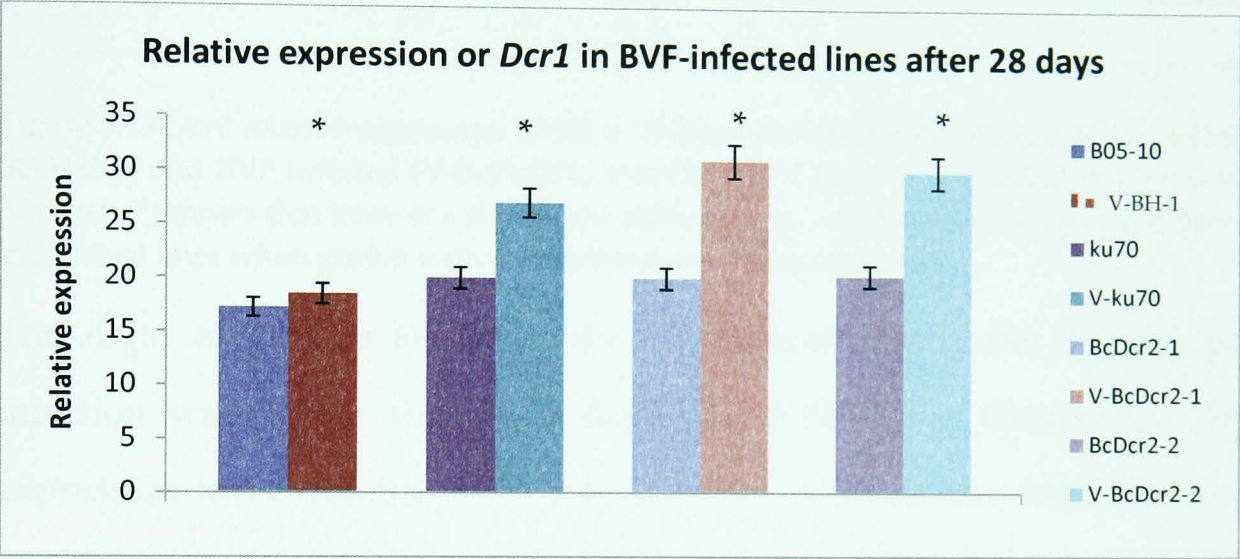


Figure 5.17 Relative expression levels at 28 days for *B. cinerea* *Dcr1* for uninfected (B05-10, *ku70*, BcDcr2-1, BcDcr2-2) and BVF infected (V-BcDcr2-1, V-BcDcr2-2, V-BH-1 and V-*ku70*) *B. cinerea* lines. Asterisk (*) shows that there is a significant difference ($p : <0.05$) in *Dcr1* expression between individual lines when grown with and without mycovirus infection.

The *Dcr2* expression levels for the BVF-infected lines were similar to *Dcr1* expression levels, which increased to levels higher than the uninfected lines. (Fig 5.18). The increase in *Dcr2* expression was also more apparent in the *ku70* mutant. The *Dcr2* mutants on the other hand, showed no signal for *Dcr2*, as expected.

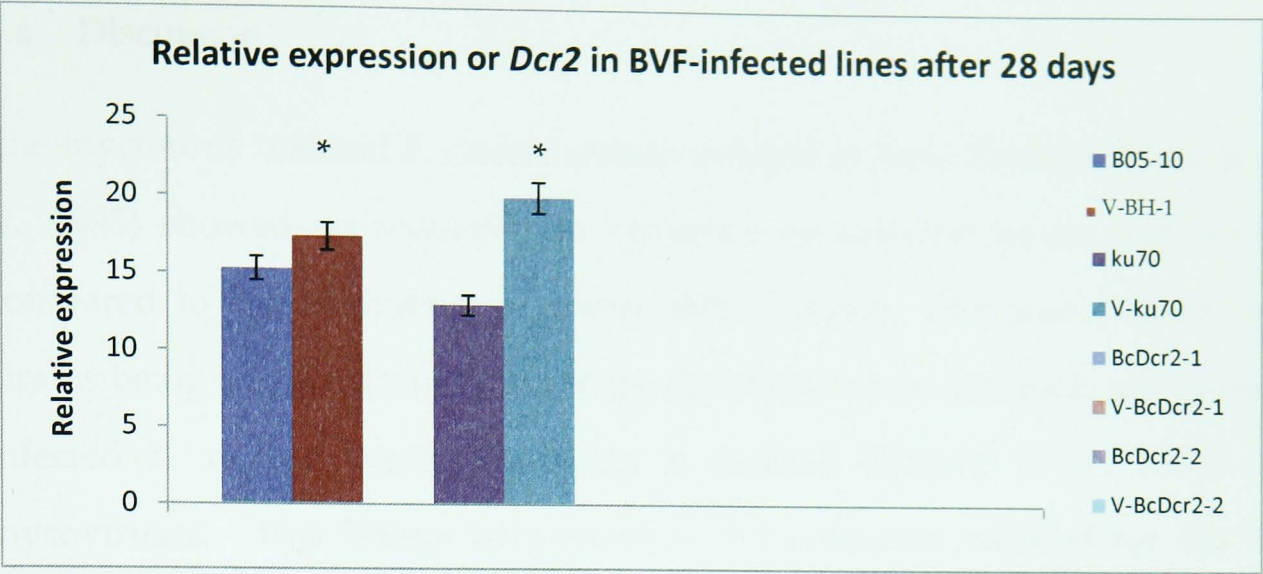


Figure 5.18 *Dcr2* relative expression levels at 28 days for uninfected (B05-10, ku70, BcDcr2-1, BcDcr2-2) and BVF infected (V-BcDcr2-1, V-BcDcr2-2, V-BH-1 and V-ku70) *B. cinerea* lines. Asterisk (*) shows that there is a significant difference ($p < 0.05$) in *Dcr2* expression between individual lines when grown with and without mycovirus infection.

The *Argo1* expression levels for the BVF-infected lines after 28 days post-infection was higher than at 7 days (Fig 5.19). Even though the *Argo1* expression levels for the BVF- infected lines were always higher than the uninfected lines, the increase in expression was more noticeable in the lines which have had the *ku70* gene disrupted.

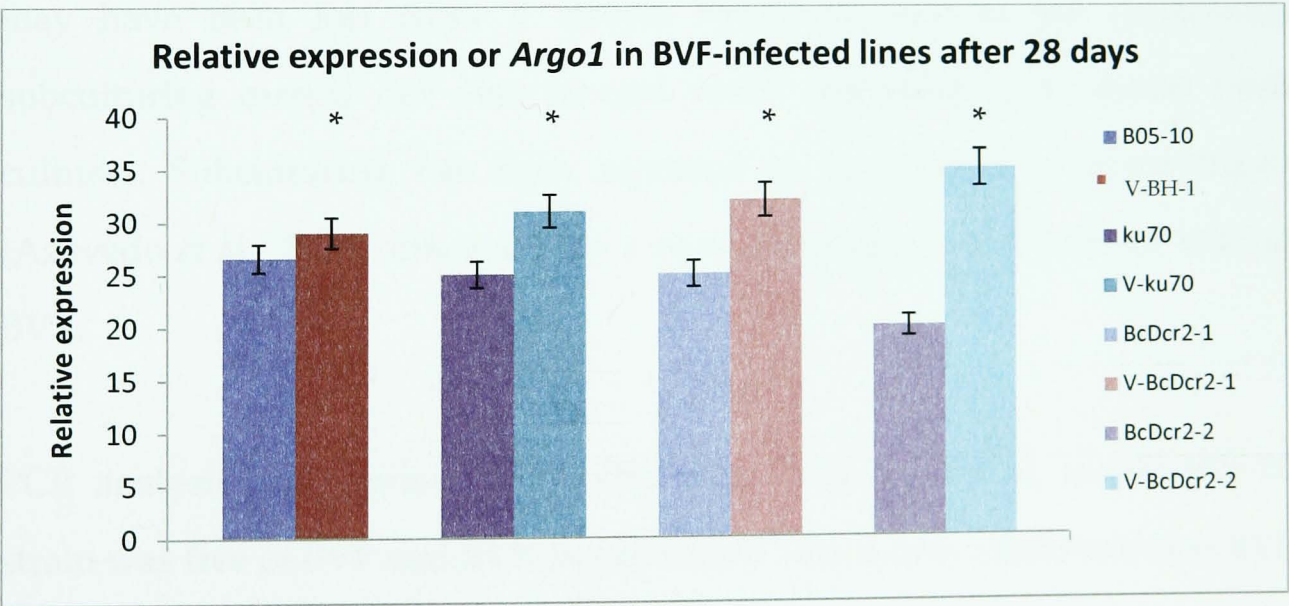


Figure 5.19 Relative expression levels at 28 days for *B. cinerea* *Argo1* for uninfected (B05-10, ku70, BcDcr2-1, BcDcr2-2) and BVF infected (V-BcDcr2-1, V-BcDcr2-2, V-BH-1 and V-ku70) *B. cinerea* lines. Asterisk (*) shows that there is a significant difference ($p < 0.05$) in *Argo1* expression between individual lines when grown with and without mycovirus infection.

5.4 Discussion

The mycovirus infected *B. cinerea* strains isolated in New Zealand (Howitt *et al.*, 1995) showed no reduction in virulence on selected leaves and fruits compared to the laboratory *B. cinerea* B05-10 strain. This was despite the strains being isolated from different geographical areas, and each mycovirus infected *B. cinerea* strain containing a cocktail (Howitt *et al.*, 1995) of mycoviruses. This brings into question the collection method for the *B. cinerea* strains which may have relied on the selection of highly visible and virulent strains.

PCR analysis of RH106-10 confirmed that BVF was present while BVX was no longer detectable by PCR analysis. The similarity in morphology for BVF and BVX meant that the flexuousrod-shaped mycoviruses observed by using the transmission electron microscope could not distinguish the two mycoviruses. It was then presumed, that only BVF was present. This mycovirus strain was first reported by Howitt *et al* in 1995, so the strain has been maintained in the laboratory for at least 15 years. The mycovirus BVX may have been lost from *B. cinerea* RH106-10 due to the continuous subculturing carried out over several years' maintenance to obtain fresh cultures. Subculturing has been reported to cure fungi of mycoviruses (Azevedo *et al.*, 2000), this could be a plausible reason for RH106-10 lacking BVX.

PCR analysis was carried out to determine whether the *B. cinerea* B05-10 strain was free of BVF and BVX mycoviruses. The results confirmed that BVF and BVX were not detectable, making this strain suitable for mycovirus transmission experimentation requiring a clean host.

The titre levels of BVF over 72 h were not consistent in the *B. cinerea* RH106-10 host. Inoculation of the fungus in liquid growth media which allows new hyphal growth may have been associated with the steady reduction in BVF titre levels. Although the mycovirus was able to replicate to higher levels again, the initial reduction in BVF mycoviral levels was possibly caused by the fungus trying to eliminate the mycovirus. Since the absolute levels for qPCR were not determined, there is also the possibility that *B. cinerea* β -tubulin expression levels fluctuated, altering the apparent relative BVF titre levels. Nonetheless, there is also the likelihood that the reduction and increase in BVF titre levels relates to a particular morphology stage to which the mycovirus prefers and adapts to overtime.

Although no obvious change in morphology was observed with the mycovirus-infected *B. cinerea* strains, this does not eliminate the possibility that the mycoviruses are conferring physiological traits to the fungal host. To ascertain this possibility, the *B. cinerea* RH106-10 strain was investigated.

Apart from the apparent loss of BVX from RH106-10 reported here, there have been no other reports of *B. cinerea* being cured of mycoviruses. This work has shown that curing BVF from RH106-10 by hyphal tip subculturing and exposure to plates containing cycloheximide concentrations up to 25 $\mu\text{g ml}^{-1}$ incubated at 25 °C was not effective. Curing using cycloheximide has also been reported to be ineffective in several other fungi. In separate studies on *Fusarium oxysporum*, one having dsRNA molecules ranging from 0.7–3 kb and the other 2.2–4 kb isolates were subcultured onto plates with cycloheximide concentrations up to 280 $\mu\text{g ml}^{-1}$ for 14 days produced no cured lines (Sharzei *et al.*, 2007; Kilic and Griffin, 1998). The entomopathogenic fungus *Metarhizium flavoviride* resulted in no cured strains

after treatment with elevated incubation temperatures, exposure to cycloheximide and single conidial subcultures (Martins *et al.*, 1999). The yeast *Yarrowia lipolytica* was also not cured by cycloheximide or by 5-fluorouracil which inhibits thymidylate synthase the enzyme used to generate thymidine monophosphate (Treton *et al.*, 1987). This shows that curing fungi using cycloheximide is not always possible. The apparent reason could not be elucidated because comparisons between different reports could not be made due to different fungal host and mycoviruses or dsRNA elements. Future curing attempts for *B. cinerea* RH106-10 may require more hyphal tip subculturing in addition to any other curing approaches that may become available.

Since it is not possible to determine the impact BVF has on *B. cinerea* RH106-10, there was a need to infect BVF into mycovirus-free strains. For this work, evidence of transfection of BVF as partially purified mycoviruses was not detectable by PCR analysis in the recipient host. The reason for this could be that either mycoviral transmission did not occur due to the low titre of infection particles in the suspension including lack of familiarity with technique, or mycoviral transmission did occur, but the recipient host B05-10 mounted an effective defence to control mycovirus infection to a level undetectable by PCR. The former could have been the possible reason since mycoviral transmission was later successful by protoplast fusion and anastomosis in introducing BVF into various derivatives of B05-10. The partial purification of mycoviruses has been successful in *B. cinerea* (Howitt *et al.*, 2006).

The BVF mycovirus was introduced into *B. cinerea* lines which included both of the *Dcr2* mutants. BVF- infected *B. cinerea* lines showed no significant

difference in growth rate and virulence compared to uninfected lines. This is unlike *C. parasitica* which showed notable reduction in mycelial growth when infected with CHV1 (Robin and Heiniger, 2001).

Despite there being no alteration in phenotype, the presence of BVF does seem to alter the relative expression levels of *Dcr1*. At 7 days, the BVF – infected lines showed a reduction in *Dcr1* expression, especially in the two BVF-infected *Dcr2* mutants, lines which also lacked the *ku70* gene.

Dcr2 expression also showed reduction in expression in the BVF-infected lines, with a stronger reduction in the BVF-infected *ku70* mutant line. There was no *Dcr2* expression in both *Dcr2* mutants as expected.

Argo1 expression levels for the BVF-infected lines were different to *Dcr1* and *Dcr2*, having an increase in expression when BVF was present.

This is not the first report of the RNA silencing machinery being targeted for suppression by viruses. The Red clover necrotic mosaic virus was reported to interfere with the accumulation of small RNAs (Takeda *et al.*, 2005). Suppression of the RNA silencing machinery required the virus to produce two replication proteins (p27 and p88). Also, the flock house virus was able to suppress the RNA silencing machinery in *Drosophila* (Li *et al.*, 2002). The virus encoded the B2 suppressor protein which inhibited the accumulation of siRNA for the target of interest. For *Aspergillus*, the *Aspergillus* virus 1816 suppressed RNA silencing activity (Hammond *et al.*, 2008), although the mechanism responsible for suppression has not been identified. The ability of these viruses to suppress the RNA silencing mechanism was carried on to the next generation as long as the viruses were present in their hosts.

By 28 days, the expression levels for *Dcr1* in the BVF infected lines had recovered, and were higher than uninfected *B. cinerea* lines. The increase was more apparent in lines which lacked the *ku70* gene.

Similar to *Dcr1*, the *Dcr2* expression levels also increased in the BVF-infected lines by 28 days, a stronger increase was noticed for lines derived from the *B. cinerea* *ku70* mutant. Although *Dcr2* was absent in the *Dcr2* mutants, *Dcr1* expression remained the same as with normal expression. This suggests that 'normal' levels of *Dcr1* are sufficient for controlling any potential over expression of the mycoviruses.

The expression levels for *Argo1* remained higher than uninfected lines just as seen at 7 days. But similar to the expression of *Dcr1* and *Dcr2*, the increase in expression was stronger in lines which had *ku70* mutant backgrounds.

The BVF titre levels in culture were found to vary, when it was measured in a comparatively short time course. This could have been caused by the cyclic expression of the mycoviruses competing with the cyclic response of the RNA silencing mechanism. This could possibly explain the qPCR results at day 7 and 28, showing that the cultures have not become stable, but that it is simply at a different point within the cycle of mycovirus growth and the fungal response.

This work has shown that the mycovirus BVF does not confer any significant phenotype traits to *B. cinerea* that would affect morphological growth or virulence. Although it would appear that there was suppression of the RNA silencing genes 7 days post-infection. This apparent suppression of silencing

is only transient, at 28 days the silencing machinery is induced and therefore may be impacting on viral titre.

6 Discussion

6.1 Background

The gray mould fungus, *B. cinerea*, attacks over 200 crops world wide (Williamson *et al.*, 2007), and most of the crops targeted by the fungus are highly marketable fruits and vegetables, where any reduction in visual appearance impacts severely on value. The fungus's ability to survive at temperatures from 0-30 °C allows it to adapt to a wide range of environments across the world (Howitt *et al.*, 2001), making finding a control difficult. Although there are effective cultural and chemical controls presently used for the control of *B. cinerea*, these options are not entirely economical (Son *et al.*, 2002), and the chemical methods are likely to be short-lived given the frequency with which resistance occurs.

There is therefore much interest in the use of biological control to provide long-term suppression of this disease. For the chestnut blight pathogen *C. parasitica*, the fungus is controlled in America and Europe by the debilitating mycovirus CHV1 (Robin *et al.*, 2009). The mycovirus causes fungal hypovirulence by down regulating the fungal host's genes including *Crp*, a gene that encodes a cell wall hydrophobin (Kazmierczak *et al.*, 1995). This promising use of a BCA to control a fungus could hopefully be adopted for *B. cinerea*, since the use of mycoviruses as a BCA would be a more environmentally safe and economical option. For this work, the potential of the ssRNA BVF mycovirus as a BCA was examined. But before transfecting the mycovirus into *B. cinerea*, it was important to learn more about the active *B. cinerea* cellular defence system (Patel *et al.*, 2008), RNA silencing.

In *Eukaryotes*, the RNA silencing mechanism has been reported to protect the organism from viral RNA and endogenous elements (Plasterk and Ketting, 2000). RNA silencing was first identified for filamentous fungi in *N. crassa*. Three *N. crassa* mutants QDE-1, QDE-2 and QDE-3 which lacked an *RdRp*, an *Argonaut* and a *Helicase* gene respectively, were not active in RNA silencing (Cogoni and Macino, 1997; Fagard *et al.*, 2000; Cogoni and Macino, 1999), but the situation in *B. cinerea* was unknown.

6.2 Dicer gene disruption

Analysis of the available genome sequences for *B. cinerea* identified two Dicer genes in *B. cinerea*; *Dcr1* and *Dcr2*, and 4 Argonauts. The *B. cinerea* Dicer genes were selected for gene disruption because in filamentous fungi, the proteins encoded by these genes were reported to be involved in the initial stages of RNA silencing (Catalanotto *et al.*, 2004), and there is minimal likelihood of genetic redundancy. Although the use of restriction enzymes for construction of Dicer disruption plasmids was explored, it was not feasible due to the lack of appropriate restriction enzymes at desired sites. Therefore, the in-yeast homologous recombination approach was used (Ma *et al.*, 1987). This method proved to be relatively trouble-free when appropriately designed, and allowed multiple DNA fragments of variable sizes (e.g. 0.5 – 10 kb) to be joined simultaneously, and also meant that recombination sites were not dictated by available restriction sites.

Three plasmids were made for the disruption of *B. cinerea Dcr1* (pYES2-D1) and *Dcr2* (pYES2-D2, pXMAS-D2) based on hygromycin selection. After transforming *B. cinerea* B05-10 with pYES2-D1, 12 transformants were identified by PCR analysis to be possible *Dcr1* mutants but were contradicted

by Southern analysis. The possible explanation for the conflicting results could be that with *B. cinerea* being a multinucleate organism, even just 1 % of the DNA preparation being from nuclei with the desired targeted *Dcr1* disruption, would be enough to give a positive by PCR analysis, but insufficient to be detected by Southern analysis. The *Dcr2* disruption plasmids pYES2-D2 and pXMAS-D2 were transformed into *B. cinerea* B05-10. After several transformation attempts and despite screening over 200 transformants, no *Dcr2* mutants were identified by PCR analysis.

To eliminate the possibility that no Dicer mutants were obtained due to poor quality materials or a flaw in experimental procedure, additional control measures were taken. More *B. cinerea* transformations for targeted disruption of *Dcr1* and *Dcr2* was carried out using fresh fungal stock and laboratory materials, which still produced no Dicer mutants. Efficiency of *B. cinerea* transformations was determined using hygromycin resistant plasmids which gave normal levels for transformation efficiency. Also, *B. cinerea* transformations were carried out using Dicer disruption plasmids at BayerCrop Science laboratory in Lyon France, under the observation of Associate Professor Mathias Choquer; no targeted disruption of either Dicer gene was obtained. This eliminated the possibility that laboratory materials and procedures contributed to the lack of Dicer mutants. Whilst these results were unusual, they were not unknown.

Given that disruption in a *ku70* mutant background had been reported to increase targeting in *B. cinerea* (Choquer *et al.*, 2008), the hygromycin resistant *B. cinerea* *ku70* strain, a derivative of *B. cinerea* B05-10, was obtained, and was transformed with the nourseothricin based *Dcr2* disruption plasmid (pNDT-2). *B. cinerea* *Dcr2* was targeted first, because of the two Dicer genes present in some filamentous fungi, only the Dicer 2 gene was usually

involved in RNA silencing (Catalanotto *et al.*, 2004; Kadotani *et al.*, 2004). Two *B. cinerea* *Dcr2* mutants were successfully obtained giving a targeted transformation efficiency of 10%, similar to *A. sojae* having less than 10 % efficiency for the disruption of the *tannase* gene in a *ku70* background (Takahashi *et al.*, 2006), but rather low when compared to 100 % efficiency for the disruption of *cnd15* in the same *B. cinerea* *ku70* strain (Choquer *et al.*, 2008). Use of *ku70* should also allow *Dcr1* and *Dcr1-Dcr2* double mutants to be constructed in the future.

6.3 RNA silencing of the *bcass1* gene

To determine whether both *Dcr2* mutants were impaired in RNA silencing, it was necessary to induce RNA silencing and measure any possible effects. The *bcass1* silencing cassette was selected because *B. cinerea* transformants would show growth reduction on media lacking arginine supplementation. The silencing cassette was ligated to a basta vector to create pLOB-bar which was transformed into the *Dcr2* mutants and parent *ku70* lines. Since there was variability in the degree of silencing between individual transformants, a population of basta resistant transformants was selected for analysis, to accurately determine the frequency and strength of silencing. The *ku70* transformants showed the *bcass1* silencing effect on media lacking arginine, demonstrating that RNA silencing was active, as expected, but unexpectedly, the silencing effect was also seen for the BcDcr2-1 and BcDcr2-2 transformants grown on media without arginine, showing that silencing was active. This shows that *B. cinerea* *Dcr2* is not essential for RNA silencing.

Phylogenetic analysis for *B. cinerea* and selected filamentous fungi which have reported Dicer gene disruption and a *Basidiomycete* as the outgroup was investigated. A phylogenetic tree (Fig 6.1) was created using the 18s rRNA gene sequences from NCBI. It showed that *B. cinerea* was more closely related to *N. crassa* than to *M. oryzae*, *C. parasitica*, *A. nidulans* and *A. bisporus*.

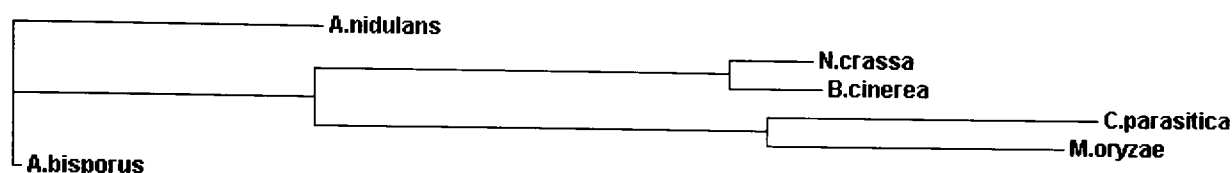


Figure 6.1 Phylogenetic tree made using the 18s rRNA gene sequences (NCBI database) of selected filamentous fungi which have had their Dicer genes disrupted, *A. nidulans* (DQ295796), *N. crassa* (AY046271.1), *B. cinerea* (AY544695), *C. parasitica* (GU993820), *M. oryzae* (GU073121) and *A. bisporus* (FJ641895) as the outgroup.

Since *B. cinerea* and *N. crassa* are closely related, and their Dicer 2 genes were not essential in RNA silencing, it could be speculated that *B. cinerea Dcr1* may have a similar role to the *N. crassa dcl-1*, that is, in processing siRNA. For *Dcr2*, its role in *B. cinerea* is of interest. Could the role of *Dcr2* be redundant in RNA silencing like *N. crassa*, or is *Dcr2* involved in another mechanism yet to be identified in *B. cinerea*?

Reviewing the reports on *Eukaryotes* having two DICER proteins each, it shows that the roles for the DICERs were not similar among the different species (Segers *et al.*, 2007; Catalanotto *et al.*, 2004). For the filamentous fungi *M. oryzae*, *C. parasitica* and *A. nidulans*, of the two DICERs present, only DICER 2 was involved in processing siRNA. For *N. crassa*, the *dcl-2* mutant was still able to produce siRNA since the small RNAs were also processed by DICER 1. So, until a *Dcr1* and a double Dicer mutant is created for *B. cinerea*, the role of *Dcr1* in RNA silencing cannot be fully understood.

Although the function of the MDL-1 (Dicer one homologue) protein in *M. oryzae* was found not to be involved siRNA production, it is thought that the protein is involved in miRNA production since disruption of *mdl-1* caused development defects such as malformed conidia (Kadotani *et al.*, 2004). Since no obvious morphological alterations were observed with the *B. cinerea* *Dcr2* mutants, *Dcr2* may have no role in processing miRNA, if indeed miRNA are involved in morphogenesis of *Botrytis*. For *Drosophila*, *dicer-1* was reported to be involved in miRNA production with the *dicer-1* mutants having abnormal phenotypes which included dark orange patches on the eyes, while *dicer-2* was involved in siRNA production (Lee *et al.*, 2004).

To induce RNA silencing in an organism, the most commonly used approaches include sense, antisense and hairpin RNA constructs (Smith *et al.*, 2000). The insertion of the constructs into the host chromosome would normally be by ectopic insertion, since targeted insertion would not be required, allowing a sufficient population of silenced lines to be obtained. For *B. cinerea*, comparisons between sense and antisense RNA constructs found antisense RNA to be more effective in inducing RNA silencing (Patel *et al.*, 2008). This may have been caused by the formation of dsRNA molecules between the endogenous sense RNA and antisense RNA in the cytoplasm without any requirement for RdRp.

Despite the numerous studies using silencing in fungi, few if any, have studied levels of the silencing genes expression. QPCR determined the relative expression levels of the RNA silencing genes *Dcr1*, *Dcr2* and *Argo1* (with β -tubulin as control) for the selected *bcass1* silenced and unsilenced lines. The expression levels for *Dcr1* showed an increase when *bcass1* silencing was active, with a further increase when grown on media lacking arginine supplementation. *Dcr2* expression also increased in the silenced

line, with the increase in silencing more greater when the silenced line was grown on media lacking arginine supplementation. Similar to *Dcr1* and *Dcr2* expression levels, *Argo1* showed an increase in expression when *bcass1* silencing was active, with a further increase when grown on media without arginine supplementation.

The qPCR results show that for *B. cinerea*, there was an increase in *Dcr1* and *Dcr2* expression when silencing was active, and a further increase when grown on media lacking arginine supplementation. These increases could be a response of the RNA silencing machinery to the possible increase in *bcass1* transcripts to cater for the lack of endogenous arginine production.

Also, it was noticed that there was no significant increase in *Dcr1* expression in either *Dcr2* mutants, to compensate for the loss of *Dcr2*. This may imply that the two Dicer genes are fully redundant in RNA silencing, or *Dcr2* is not involved in RNA silencing.

In this study, four different selection markers have been successful in creating antibiotic/herbicide resistant transformants; for *B. cinerea*, hygromycin, basta and nourseothricin (Liu *et al.*, 2008; Choquer *et al.*, 2008). The only other dominant marker reported for *B. cinerea* transformation is phleomycin resistance. This lack of markers may be a limiting factor if further molecular modifications are required for the *Dcr2* mutants. Hopefully, more effective selective markers may become available for *B. cinerea* studies. For this work, the basta marker was found to be more reliable for the selection of positive transformants when compared to hygromycin and nourseothricin.

6.4 *B. cinerea* infection with mycovirus BVF

Mycovirus-infected fungi have been shown to confer hypovirulence to their fungal host (Robin and Heiniger, 2001). Therefore, the virulence levels for selected mycovirus infected *B. cinerea* strains from New Zealand and the B05-10 lab strain (control) were determined by plant and fruit bioassays. The results showed no difference in virulence between the mycovirus infected strains and control. This could either be that the mycoviruses present in these selected mycovirus-infected *B. cinerea* strains are mild (latent) or, the RNA silencing mechanism in *B. cinerea* is able to control the mycoviruses.

Botrytis cinerea has numerous vegetative compatibility groups (VCGs) which may limit the use of mycoviruses as biological control agents. This limitation could be overcome if the mode for mycoviral dispersion in the field is by mating of the microconidia and sclerotia which occurs independently of VCGs (Glass *et al.*, 2000). Also, it would be ideal that the possible biological control agent to be vegetatively compatible with the *B. cinerea* strains associated with the targeted crop. It may not be acceptable to deliberately release an infected *Botrytis* strain in the field even if it has low virulence due to hypoviruses.

There are circumstances where infection and disruption of normal development in a fungus by a mycovirus is to be avoided. Examples would be infection of the edible mushroom *A. bisporus* by MVX and La France mycoviruses which cause loss of mushroom production (Grogan *et al.*, 2003; Romaine and Goodin, 2002). There have been reported resistance of mushrooms to mycoviral infection, although the cause was possibly due to vegetative incompatibility during anastomosis, which reduces the likelihood of mycoviral movement (Fletcher, 1992).

The RNA silencing machinery has been reported to work in *A. bisporus* (Costa *et al.*, 2008). Therefore, it would be of interest to introduce RNA silencing inducing constructs into the mushrooms that would target the *A. bisporus* mycoviruses. This would trigger RNA silencing of the selected mycoviruses, and possibly reduce or eliminate mycovirus infection in *A. bisporus*.

Several agricultural virus resistant crops such as squash (Fuchs *et al.*, 2004) and papaya (Lius *et al.*, 1997) have been genetically modified to protect against virus infection. For the transgenic *Carica papaya* cultivars, the coat protein (CP) gene of the ssRNA positive sense Papaya ringspot virus (PRV) was introduced into *C. papaya* to create resistant cultivars to PRV infection (Lius *et al.*, 1997). The CP from the mild PRV HA5-1 strain was engineered into the plants on the basis that PRV-CP was present during viral infection (Tripathi *et al.*, 2008).

The potential of the ssRNA mycovirus BVF as a BCA was investigated. To understand the impact the mycovirus is having on its host *B. cinerea* RH106-10, an attempt was made to cure the fungus of the mycovirus, so the fungus could be studied. In the attempt to cure RH106-10 of BVF, the strain was subjected to hyphal tip subculturing and exposure to cycloheximide concentrations up to 25 $\mu\text{g ml}^{-1}$. After several repeated subculturing, BVF remained present. The use of cycloheximide has also been ineffective in curing mycoviruses in other fungi too (Aoki *et al.*, 2009). To date, there have been no reports explaining the possible reason why curing using cycloheximide has not worked for some fungi. This is the first report of an attempt to cure an ssRNA mycovirus from *B. cinerea* to the author's knowledge. Future attempts in curing BVF from RH106-10 should include hyphal tip subculturing for more than 8 weeks, and to investigate the

potential of the antiviral drug Ribavirin, which interferes with viral replication (Park *et al.*, 2005), and emetine (Kim *et al.*, 2006), which disrupts the viral translocation process in curing mycoviruses in *B. cinerea*. These possible mycovirus curing chemicals have not been applied to *B. cinerea*, but have been used on other filamentous fungi (Park *et al.*, 2005; Kim *et al.*, 2006).

To establish whether BVF could alter the fungal phenotype in a mycovirus-free *B. cinerea* host, BVF was transfected by protoplast fusion and anastomosis into the hygromycin resistant lines. These transfection approaches were equally effective in introducing the mycoviruses into *B. cinerea*, although transfection by anastomosis was less laborious. The BVF infected BH-1, both *Dcr2* mutants and the parent *ku70* lines showed no apparent difference in growth rate on MEA media, and in virulence in plant bioassays, compared to the virulent lab strain B05-10.

At the molecular level, the presence of BVF affected the relative expression levels of the RNA silencing genes (*Dcr1*, *Dcr2*, *Argo1*) for the BVF-infected lines. At 7 days post-infection, *Dcr1* expression levels decreased for most of the BVF-infected lines, with both of the *Dcr2* mutants, which also lack the *ku70* gene, having a greater decrease. The BVF-infected infected lines showed a strong decrease in *Dcr2* expression compared to the uninfected lines, with the decrease more apparent in the *ku70* mutant. There was no *Dcr2* signal for both of the *Dcr2* mutants, as expected.

The qPCR results suggest there was mycovirus-mediated suppression of the Dicer genes after 7 days post-infection for the BVF-infected lines compared to the uninfected lines. This could mean that the presence of BVF reduced the expression of the Dicer genes, and that the suppression of expression was

greater in lines which lacked the *ku70* gene. However, despite apparent Dicer suppression, *Argo1* expression level, unlike the Dicer genes, showed an increase in expression for the BVF-infected lines, regardless of genetic background. The increase in *Argo1* expression showed a similar pattern in *Argo1* expression in the *bcass1* silenced lines, having an increase in expression for the transformed lines compared to untransformed lines. This shows that the presence of the mycoviruses in *B. cinerea* cannot suppress *Argo1* expression.

It is not clear which, if any, of the BVF proteins are responsible for this suppression. BLAST search using the BVF protein sequences showed no similarity to known viral suppressor proteins of the RNA silencing mechanism which included the p29 and HC-Pro components encoded by the *C. parasitica* mycovirus CHV1-EP713 and the PVX virus respectively (Segers *et al.*, 2006). The expression of p29 reduced conidiation in its host (Craven *et al.*, 1993), while the expression of HC-Pro in *Nicotiana benthamiana* caused tumour formation (Anandalakshmi *et al.*, 2000). In *C. parasitica*, suppression of the genes *Cpkk1* and *lac1* from the signalling pathways by the mycovirus CHV1 affected fungal development (Turina *et al.*, 2006; Parsley *et al.*, 2002). The genes from the signalling pathway were not investigated in mycovirus infected *B. cinerea* because the fungus did not show any altered phenotype.

By 28 days post-infection for the BVF-infected lines, the *Dcr1* expression levels showed recovery, with the increase in expression greater in the *ku70* mutant background. Similar to *Dcr1*, the *Dcr2* expression levels also increased, with the increase stronger in the *ku70* mutant. The *Argo1* expression levels for the BVF-infected lines remained higher than uninfected lines. For the BVF-infected *B. cinerea*, the recovery of the suppressed RNA

silencing genes showed that the fungus was seemingly able to counter initial gene suppression by 28 days. Although BVF was not eliminated since the mycovirus remained detectable by qPCR, the mycoviral titre levels were not steady for days 7 and 28 post-infection. This work shows that *B. cinerea* was able to inhibit BVF from conferring any possible traits that would have caused altered phenotypes.

Viruses that have RNA silencing suppressor proteins have the advantage of suppressing the hosts RNA silencing mechanism that targets RNA viruses (Chapman *et al.*, 2004). Virus mutants without the suppressors were not able to produce high virus numbers and could not spread effectively in the host (Vance and Vaucheret, 2001). The suppressor proteins reported in plant viruses include the P1/HC-Pro (Kasschau *et al.*, 2003), 2b (Goto *et al.*, 2007), p19 and p21 (Chapman *et al.*, 2004). The turnip mosaic virus (TuMV) encodes the P1/HC-Pro protein which induced growth defects in *Arabidopsis*, because the suppressor protein interfered with miRNA processing (Kasschau *et al.*, 2003). TuMV infected-*Arabidopsis* showed severe stunting consistent with the increase in P1/HC-Pro mRNA production. The 2b suppressor encoded by the cucumber mosaic virus (CMV) was shown to suppress the RNA silencing machinery by binding siRNA. In contrast, 2b was weak in binding miRNAs (Goto *et al.*, 2007), this observation was supported by minor developmental changes shown when *Arabidopsis* was infected with viruses encoding 2b (Chapman *et al.*, 2004). The suppressor p19 encoded by the Beet yellow virus and p21 encoded by the Tomato bushy stunt virus were efficient in suppressing the host RNA silencing machinery by binding the siRNAs and miRNAs (Goto *et al.*, 2007). These suppressor proteins are distinct from each other, implying that they have evolved from different paths (Vaucheret *et al.*, 2006). But the suppressors were similar in that they all targeted the

siRNA/miRNA before the short RNA could be incorporated into the RISC complex (Chapman *et al.*, 2004).

Mycoviruses that suppress the RNA silencing mechanisms in eukaryotic hosts do not always weaken the host (Goto *et al.*, 2007). Therefore, identifying a mycovirus that is able to suppress the RNA silencing mechanism and confer hypovirulence would be an ideal biological control agent for *B. cinerea*. A mycovirus for the control of *B. cinerea* with such traits would either be obtained by sampling in the field or by molecular engineering new mycoviruses. Sampling in the field would be time consuming, while engineering a mycovirus would require selecting sequences that encode a RNA silencing suppressor from fungal or plant viruses that are effective in *B. cinerea*, and using hypovirulent *B. cinerea* mycoviruses.

When engineering a complete mycovirus, it may be preferable to choose sequences which encode proteins that evade detection by the host's RNA silencing mechanism rather than RNA silencing suppressors, since resources for the mycoviruses would be used for replication and dispersal, rather than using them for suppression of the fungal defence system. Also, if the eukaryotic host RNA silencing mechanism is suppressed by the mycoviruses, the fungal host may adapt and find other means to control mycovirus infection, thus, eliminating the effectiveness of the virus suppressor proteins.

Observing the mycoviral titre levels within the short time-course and the expression levels for the RNA silencing genes at day 7 and 28 in the BVF-infected lines, there remains the possibility that both may be cyclical. The increase in BVF titre may trigger a silencing response, reducing the titre

levels, then the mycovirus responds by suppressing the RNA silencing allowing an increase in BVF activity, and the cycle continues. This could imply that by day 7 or 28, the RNA silencing genes were not stable, but was just a point within the cycle of mycovirus growth and the fungal response. This would require a far more detailed study to investigate it properly.

Unfortunately for this work, expression analysis for some of the RNA silencing genes was by necessity in *ku70* mutant backgrounds, such as the *Dcr2* mutants and *B. cinerea* *ku70*. The significance of the increase in expression of the RNA silencing genes in the silenced lines, and suppression and subsequent recovery of the Dicer genes in the BVF-infected lines being more prominent in lines lacking *ku70* was is not known. Therefore, the possibility that *ku70* is playing a role in the regulation of RNA silencing cannot be eliminated. In humans, the *ku70* antigen has been identified as part of the human DNA helicase II, a DNA unwinding enzyme (Tuteja *et al.*, 1994). For mice, the *ku70* antigen was found to be associated with DNA helicase II activity and RNA processing (Zhang *et al.*, 2004). This could mean that *ku70* is involved in transcriptional or post-transcriptional regulation, although this has yet to be confirmed in filamentous fungi. Complementing the *ku70* back into both *Dcr2* mutants, would show whether the loss of *ku70* had any effect on the expression of the RNA silencing genes.

The expression levels for *Dcr1*, *Dcr2* and *Argo1* for the *bcass1* silenced lines and the BVF-infected lines were compared. Although the pattern of expression for the RNA silencing genes was similar between silenced lines and BVF-infected lines by 28 days, there was a greater increase in expression of *Dcr1*, *Dcr2* and *Argo1* for the BVF-infected lines. This showed that there

was a stronger affect on the RNA silencing machinery when mycovirus BVF was present, than simply silencing of an endogenous gene.

Botrytis cinerea has four Argonaut genes. Preliminary qPCR work showed that the expression of these genes was the same among the different *B. cinerea* backgrounds (B05-10, ku70, *Dcr2* mutants, *bcass1* silenced, BVF infected lines, so only *Argo1* was selected for qPCR analysis in this work. In *C. parasitica*, only one (*Agl2*) of the four Argonauts was required for RNA silencing (Sun *et al.*, 2009). The *Agl2* mutant was found to be vulnerable to mycoviral infection. Sequence comparisons showed that *C. parasitica* *Agl2* was not similar with *B. cinerea* *Argo1*. For *Arabidopsis*, two out of ten Argonauts were found to be involved in reducing mycoviral titre (Harvey *et al.*, 2011). AGO1 and AGO2 *Arabidopsis* mutants were more susceptible to Turnip crinkle virus and Cucumber mosaic virus infection compared to the wild type (Morel *et al.*, 2002; Harvey *et al.*, 2011).

For future work, it would be of interest to investigate the introduction of BVF and other viruses into a *bcass1* silenced lines to determine whether the mycoviruses are able to suppress the phenotypes caused by RNA silencing in *B. cinerea*. If the mechanism is suppressed by BVF, the *bcass1* silenced lines would possibly show no reduction in growth on Gamborg media without arginine supplementation.

Future investigations may try to identify which protein encoded by BVF is responsible in regulating the expression of the Dicer genes within the initial stages of infection. This could be done by cloning each of the three open reading frames of BVF and transforming them separately into mycovirus-free *B. cinerea*, and studying their effects in terms of ability to suppress silencing or to support elevated viral titre.

From this work, it could be concluded that the ssRNA mycovirus BVF may not be a suitable candidate as a biological control agent for *B. cinerea*. This of course does not eliminate the possibility of mycoviruses yet to be identified from the field to be viable BCAs that could control and help reduce the huge economical losses caused by the fungus.

Since the mycovirus BVF was not able to confer any obvious hypovirulent traits, this brings into question the collection method for the mycovirus-infected strains used in this work. The collection methodology may have been biased in selecting highly visible *Botrytis* symptoms on plant material, and thereby not including possible mycovirus-infected fungi with altered phenotypes or reduced virulence which may be harder to identify.

There have been reports of a *B. cinerea* mycovirus and a mitovirus that conferred hypovirulence to *B. cinerea*. A dsRNA mycovirus in *B. cinerea* CCg425 was able to confer hypovirulence to the fungus (Castro *et al.*, 2003), to date, no report has been made of its potential as a BCA. In the *B. cinerea* CanBc-1A strain, a 3 kb dsRNA mitovirus was reported to reduce mycelial growth (Zhang *et al.*, 2010). Studying these viral elements and identifying their potential at the commercially level would be of interest for future work.

Ideal biocontrol mycoviruses would be those that would be able to spread between the different strains of *B. cinerea*, withstand the range of environmental pressures, such as temperature and humidity, and combat against the cellular defences in the fungal host. Preferably, the mycoviruses could be fungicide resistant when applied in fields that practice integrated pest management. Monitoring the biocontrols in the field would be important not to introduce the mycoviruses into untargeted organisms to

reduce any possible threat the biocontrol may have on natural ecosystems. Also, care must be taken that the biocontrol mycoviruses do not increase fitness of the *Botrytis* strains and make them more virulent.

In filamentous fungi to date, only dsRNA mycoviruses (Sun *et al.*, 2009) have been able to weaken the fungus. But these are the most studied so it could be coincidence. The possibility that in fungi, hypovirulent mycoviruses could only be found as dsRNA molecules, but not ssRNA molecules, cannot be assessed due to the low number of mycoviruses indentified so far. Mycoviruses which have dsRNA genomes infect a wide range of hosts including bacteria, fungi and plants (Ghabrial and Suzuki, 2009).

More needs to be understood for the reason mycoviruses, which require a host for replication and survival, would weaken or even kill the host. It is understandable in the case of the mycovirus infected-killer yeast which only allows the survival of yeast colonies infected with the dsRNA molecules (Reiter *et al.*, 2005). There could be a possibility that the conferred hypovirulent traits by some mycoviruses to their hosts is unintended (Whitham *et al.*, 2006), but is of great benefit in agriculture as BCAs.

There is a report where the presence of the mycovirus is very important for the symbiotic relation between a mycovirus, a fungus and a plant for thermal tolerance (Marquez *et al.*, 2007). It showed that the plant host was only heat tolerant when the mycovirus infected fungus was present in the monocot host.

Several mycoviruses including those isolated from *B. cinerea* (Wu *et al.*, 2007) and *Sclerotinia sclerotiorum* (Pearson *et al.*, 2009) have been shown to disrupt normal developmental growth of the host fungus, but few have been used commercially as BCAs. Due to limited reports in this area, it is not clear

whether mycoviruses that confer hypovirulence are not feasible as biological control agents because of the high number of vegetative incompatibility groups for some fungi. These groups are thought to act as a pathogen defence system (Paoletti and Saupe, 2009) by restricting the movement of cytoplasmic and genetic elements between incompatible groups. But with the proven success of CHV1 in controlling *C. parasitica*, it shows that it is possible to have a mycovirus control a fungus (Ding *et al.*, 2006).

After identifying biocontrol mycoviruses in the laboratory for *B. cinerea*, the next step would be to introduce the mycoviruses into the field. A possible approach could be the use of an incapacitated fungus carrying the biocontrol mycovirus. This would allow the spread of the hypovirulent mycoviruses between the different strains of *B. cinerea* by anastomosis without the carrier fungus being a threat to agriculture. For the control of the chestnut blight, the hypovirulent strain of *C. parasitica* successfully spread the CHV1 mycovirus by anastomosis (Nuss, 1992). With the fast advances in biotechnology, there would hopefully be a way to infect virulent strains of *B. cinerea* by directly spraying the mass produced biocontrol mycoviruses onto the fungi in the field.

There remains a possibility that deletion of *B. cinerea Dcr1* would eliminate the ability for RNA silencing, and as a possible consequence, RNA mycoviruses would be able to replicate and cause effective hypovirulence. The uncertainty with the role of *B. cinerea Dcr1* can only be resolved when *B. cinerea Dcr1* mutants and double Dicer mutants are created. This work has shown that *Dcr2* is not essential for RNA silencing to function and that viral titres are not regulated by *Dcr2*.

7 References

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Appendix 1 PCR primers sets used in PCR and qPCR analysis.

1. Primers for plasmid construction

pYES2-D1

A	Fw- GAACCTTCAAGTTCCATTGTGTGGCGTATTGTGGA Rv -AGACGAAGACAAGATTGTAGGTGGAACGGCAGTAA
B	Fw -ATAATGCGATTAGTTTTTTAGCCTTATTTCTGACGTGATGAGTTAGTGTGGTATA Rv -GCCCCAATCGGGAATGCGGTCCACAGCTGC TATTATGAGATGCTATCTACTGC
C	Fw -GCAGCTGTGGACCGCATTCC Rv -GAGTGGGCGCTTACACAGTA
D	Fw- TACTGTGTAAGCGCCCACTCCACATCTCCA TGTGTCCGAATTTCTACAGACTTAA Rv -ATAGGGACCTAGACTTCAGGTTGTCTAACTACTACAAGCTTGTCAATCGAATC
E	Fw- GAATGAGTGTCCGAGAATTCAGGGATGAATACGGG Rv- ATCCCTCAATATTCATCTGATAATCTCGACCTTGGC

pYES2-D2

A	Fw -CTTGGGATACACATTTATTCTCGCGACTTTTCCTTGG Rv -TCGGACGCAAATCTTGACCAATCCGGCCCCGAATCG
B	Fw -ATAATGCGATTAGTTTTTTAGCCTTATTTCTGATGTGGAGACACTCGTATA Rv -GCCCCAATCGGGAATGCGGTCCACAGCTGCAAGTATCGGAATATTGCGTTCAGCG
C	Fw -GCAGCTGTGGACCGCATTCC Rv -GAGTGGGCGCTTACACAGTA
D	Fw -TACTGTGTAAGCGCCCACTCCACATCTCCA CCTCTACAGTCTCATTAAAGTCGTTT Rv -ATAGGGACCTAGACTTCAGGTTGTCTAACTAAATTATGAACGGCAGCAAGAGTGA
E	Fw -AATCCTTCTTTCTAGAAGTCCTCGTGTACTGTGTAA Rv -CCTCATTCCCAGTTAGGGTTTTTAGTCTAACGAATCA

pXMAS-D2

A	Fw -CCGACGTTGTGGAAGCACTCATCGGCGCATGTATG Rv -TCGGACGCAAATCTTGACCAATCCGGCCCCGAATCG
B	FwTAATGCGATTAGTTTTTTAGCCTTATTTCTCTGACATGAAATTTAGGAACTCCCCATG

C Rv -CCGAATCGGGAATGCGGTCCACAGCTGCCCTCGGCGATTCCATTCTCGACAACATCAT
 Fw -GCAGCTGTGGACCGCATTCC
 Rv- GAGTGGGCGCTTACACAGTA

D Fw- ACTGTGTAAGCGCCCACTCCACATCTCCATTAGAATCTTGCTGTCTTGCTCGCCCTCGT
 Rv- GGGACCTAGACTTCAGGTTGTCTAACTCAGCGCAGGGGAGAAGGGAGAGATTGTGAT

E Fw -CAAGCTCATGCCAATTTGTGCGCTTATCACCTGAAG
 Rv -AATCCTTCTTTCTAGAAGTCCTCGTGTACTGTGTAA

pNDT-2

A Fw -TAGAGATGTGGTAAAGCACTGTCAAGGTCT
 Rv -ACTTCGGAACCTGACACTGACCCGGATACA

B Fw- TAATGCGATTAGTTTTTTAGCCTTATTTCTCTGACATGAAATTTAGGAACTCCCCATG
 Rv -GCCCCAATCGGGAATGCGGCTCTAGAGGCCTCGGCGATTCCATTCTCGACAACATCAT

C Fw -CCTCTAGAGCCGCATTCCCGATTCTGGGCCGG
 Rv -CGGCGCGCCGAGTTAGGCAACCATGCATGG

D Fw -CATGCATGGTTGCCTAACTCGGCGCGCCGTTAGAATCTTGCTGTCTTGCTCGCCCTCGT
 Rv -GGGACCTAGACTTCAGGTTGTCTAACTCAGCGCAGGGGAGAAGGGAGAGATTGTGAT

E Fw -TAGAGATGTGGTAAAGCACTGTCAAGGTCT
 Rv -ACTTCGGAACCTGACACTGACCCGGATACA

2. Primers for Dicer analysis

Dcr1

F Fw -GAACCTTCAAGTTCATTGTGTGGCGTATTGTGGA
 Rv -GAGTGGGCGCTTACACAGTA

G Fw -GAACCTTCAAGTTCATTGTGTGGCGTATTGTGGA
 Rv - GAATGAGTGTCCGAGAATTCAGGGATGAATACGGG

Dcr2

F Fw -TAGAGATGTGGTAAAGCACTGTCAAGGTCT
 Rv -CGGCGCGCCGAGTTAGGCAACCATGCATGG

G Fw -TAGAGATGTGGTAAAGCACTGTCAAGGTCT
 Rv - ACTTCGGAACCTGACACTGACCCGGATACA

3. Primers for *Bcass1* silencing

H	Fw -ACTTTGCTTCCCTGCCGTCCAATGC
	Rv -GGCTTTGCCTTTGTGCTGCTGACTG
I	Fw -AGGCATTCCAGTCAGCAGCACAAAG
	Rv- AGCAATGGCGTTGGATTGAGGAAG

4. Primers for Mycoviruses

BVF	Fw -TTACAAGCCCTTGTTGCTTCTACA
	Rv -CGGTCATGCAGTTGTTGTTCCAGG
BVX	Fw -TCGTCTGGAACCACTTCTG
	Rv -GATGGCTGCGGTGTATTGAG

5. Primers for PCR and qPCR analysis

<i>Dcr1</i>	Fw -CCTAATACCCTCCTTCCACTAACCGCACAT
	Rv -CTACCACCGCCTATAATTTGCTATACACCT
<i>Dcr2</i>	Fw -ACGAAGAGAACGTGAATGATGAAATGTCTC
	Rv -TTCATTGTTGAAGATTCACCAGCTGCCGAG
<i>Argo1</i>	Fw -GCTACATCTGCTGCTATTGG
	Rv -GCGGATCTCCCTTTGTAAACTT
<i>β tubulin</i>	Fw -GG AAGCTT TACATGACATGAAGTTCTTATCAGAAGACG
	Rv -GG CCATGGTACTTCGTTGAGTGGATCCCTAACAACG

Appendix 2 Plasmids pYES2-19 (A) and pOliHygTrp-new used in the construction of Dicer disruption plasmids pYES2-D1, pYES2-D2 and pXMAS-D2.

